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Introduction

Metastatic mammary tumor cells frequently express high levels of the transforming growth factor β (TGF- β). Although TGF- β is a potent tumor suppressor, it can promote formation of highly metastatic tumors by stimulating an epithelial to mesenchymal transition (EMT), migration, invasion, and changes in tumor microenvironment. The molecular mechanisms of EMT, cell migration and invasion induced by TGF β are not well understood. We found that inhibition of the p38 mitogen activated protein kinase (p38 MAPK) pathway blocked TGF β -induced EMT in NMuMG mammary epithelial cells [1]. Inhibitors of p38 MAPK also blocked migration of MDA-MB-231 and 4T1 metastatic carcinoma cells [1]. These results suggest a critical role for p38 MAPK signaling in the tumor promoting activity of TGF- β . In addition, recent studies suggest that the p38 MAPK signaling pathway is important for the invasive phenotype of carcinoma cells by contributing in the activity of matrix proteases [2, 3]. Thus, the p38 MAPK pathway is a potential target for selective therapeutic intervention. The purpose of this research is (1) to examine *in vitro* the role of the p38 MAPK pathway in tumor cell motility and invasiveness, and (2) to evaluate the role of p38 MAPK in tumor growth and metastasis *in vivo*.

To study the contribution of TGF β -p38MAPK signaling in metastasis, we have used metastatic breast cancer MDA-MB-231 cell line. These cells were genetically modified to express kinase mutants of the TGF- β type I receptor (T β RI), Alk5.

In the first annual report we have presented the studies for Task 1. The completion of Task 1 was approved and some of the results were published [1, 4]. We showed that p38 MAPK inhibitors effectively blocked TGF β -induced migration of breast cancer cells and fibronectin expression. We also reported that kinase-active TGF- β type I receptor Alk5-T204D stimulates p38 MAPK activity and cell migration, whereas kinase-inactive type I and II receptors inhibit these responses. Thus, TGF- β may contribute to metastasis by increasing cell motility and changes in microenvironment, and p38 MAPK inhibitors effectively block these responses. The annual report of June 2004 describing the studies for Task 2 has been approved. In the June 2004 report we showed that constitutively active TGF- β type I receptor Alk5-T204D enhances, whereas kinase-inactive receptor Alk5-K232R reduces cell migration. Alk5-T204D increased formation of pulmonary macrometastatic lesions in an experimental metastasis model and formation of bone metastasis in orthotopic xenograft model. Alk5-T204D increases tumor cell invasion and MMP9 activity but reduces cell proliferation. p38 MAPK inhibitors block Alk5-T204D-mediated increase in MMP9 activity, tumor cell migration and invasion. Thus, these results suggested that p38 MAPK contributes to Alk5-mediated tumor invasion and metastasis by increasing MMP9 activity and cell motility.

To investigate the role of the p38 MAPK pathway in TGF- β -mediated metastasis we have prepared MDA-MB-231 cell lines expressing dominant-negative mutants of (1) TGF- β activated kinase TAK1-K63W, (2) mitogen activated kinase kinase 6, MKK6AL, and (3) p38AGF. In this annual report we present the results of *in vitro* and *in vivo* studies with these cells.

Body

According to the approved Statement of Work we performed studies stated in Task2.

Task2b: *Examine the role of p38 MAPK in metastasis using genetically engineered mammary tumor cell lines.*

The experimental design is presented in Figure 1. Human breast carcinoma MDA-MB-231 cells expressing kinase mutants and control EGFP-expressing cells were generated by retroviral infection with bi-cistronic vectors encoding the mutants and EGFP (Enhanced Green Fluorescent Protein).

Expression of EGFP is controlled by Internal Ribosome Entry Site (IRES). GFP-positive cells were selected by FACS and characterized for the expression of epitope-tagged mutants and their effects on fibronectin expression (Fig. 1) and [1].

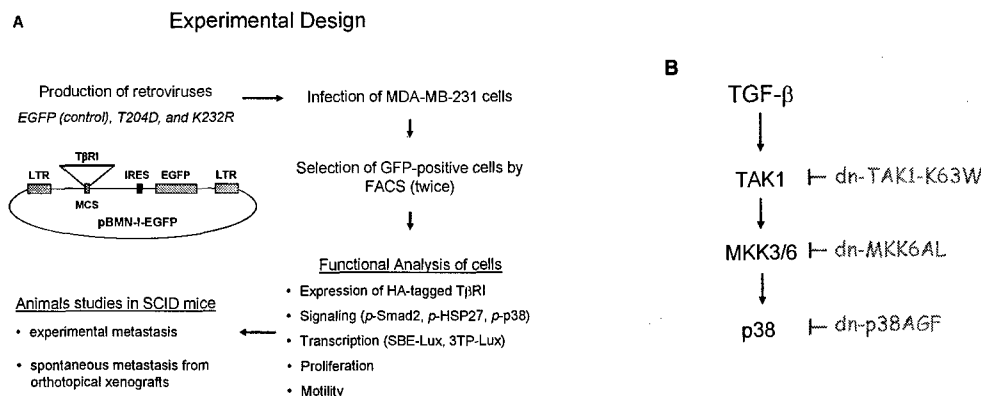


Fig. 1. (A) Kinase mutants of TGF-β type I receptor (TβRI/Alk5), and dominant-negative mutants of TAK1, MKK6, and p38 MAPK (see panel B) were subcloned in the pBMN-IRES-EGFP bi-cistronic retroviral vector. Amphitropic retroviruses were generated using Phoenix cells. EGFP-positive cells were selected after two repeats of FACS sorting. Cells were characterized for expression of mutants. TβRI and MKK6AL contain HA-tag, p38AGF has Flag-tag, and TAK1 is non-tagged.

Kinase-active Alk5-204D increased a basal phosphorylation of Smad2/3 and expression of fibronectin, an extracellular matrix protein, which is involved in tumor metastasis. Kinase-inactive Alk5-232R and TβRII-K277R reduced these responses (Fig. 1 and [1, 4]).

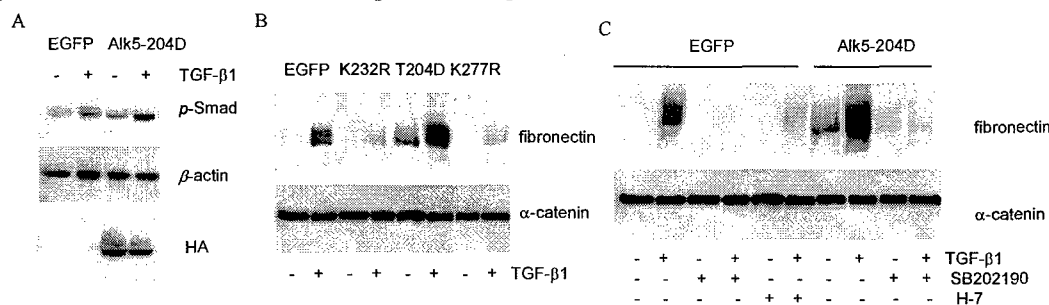


Fig. 2. Effect of kinase-mutants on phosphorylation of Smad2/3 (A) and expression of fibronectin in MDA-MB-231 cells treated with 2 ng/ml TGF-β1, and 20 μM of H-7 or 10 μM SB202190.

p38 MAPK kinase inhibitor SB202190 blocked expression of fibronectin without effect on phosphorylation of Smad2/3 (Fig. 3). Examination of transcriptional responses in the engineered cells (Fig. 4) showed that kinase-active Alk5-T204D increased by nearly 8-fold the basal activity of p3TP-Lux reporter containing a fragment of PAI-1 promoter and pSBE-Lux reporter containing twelve repeats of Smad-binding sites (described in [1]). Kinase-inactive Alk5-K232R reduced TGF-β-induced activity of the reporters (Fig. 4).

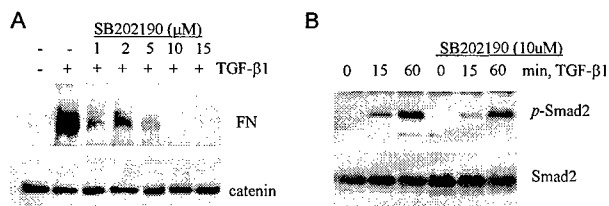


Fig. 3. Effect of p38MAPK inhibitor SB202190 on expression of fibronectin (A) and phosphorylation of Smad2/3 (B) in cells treated with 2 ng/ml TGF-β1.

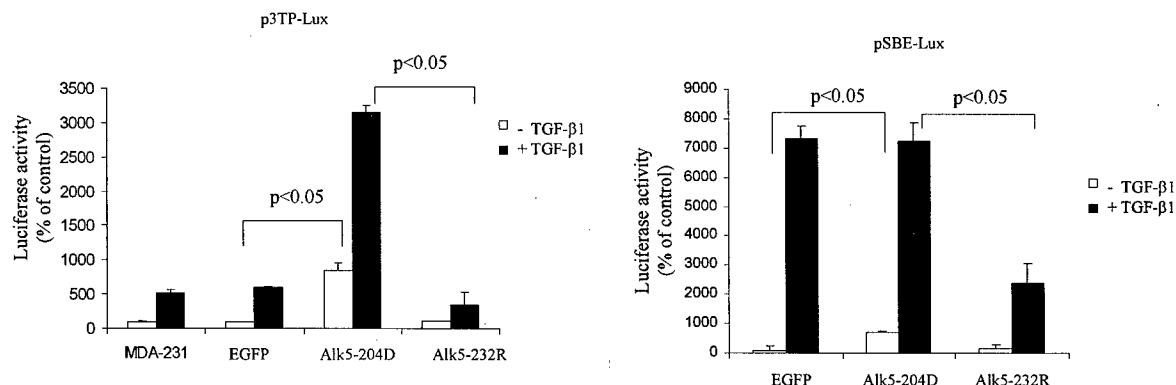


Fig. 4. Luciferase reporter activity in parental and genetically engineered MDA-MB-231 cells (see text) co-transfected with the p3TP-Lux reporter (left panel), or pSBE-Lux (right panel) pCMV-Renilla. Firefly and *Renilla* luciferase activities were measured using Promega's dual luciferase reporter assay system. Each data point represents the mean \pm S.D. of three wells. $p < 0.05$ compared to control using ANOVA test.

To address the role of TβRI/Alk5-mediated signaling in metastasis we performed experimental metastasis studies by examining formation of pulmonary macrometastatic lesions following the tail vein injection of the engineered tumor cells in SCID mice. Briefly, tumor cells (1×10^6) re-suspended in 0.1 ml of sterile Hank's solution were injected using a 28-G needle into tail vein of 8 week-old female SCID mice. Mice will be sacrificed 4 weeks thereafter. Lungs were stained with 15% Indian black ink

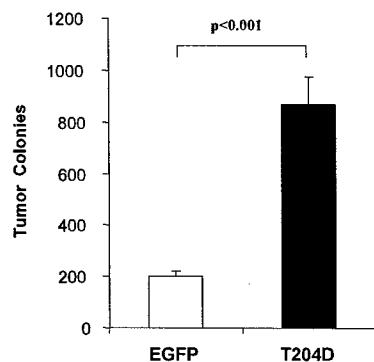


Fig.5. MDA-MB-231 cells expressing active TβRI-T204D receptor and control EGFP cells were injected in tail-vein of SCID mice (1×10^6 /mice, 8mice/group). Four weeks later mice were euthanized and lungs were stained with 15% black Indian ink. Lung surface macro-metastases were counted. $P < 0.001$ compared to control using ANOVA test.

via trachea and metastatic lesions on lung surfaces were counted. These studies showed a 4-fold increase in lung surface metastases in Alk5-T204D-injected mice (Fig. 5).

Tumor take, growth, and formation of metastases to lungs, bone, liver, and lymph nodes by the control and genetically modified MDA-MB-231 cells were examined in a spontaneous metastasis model [7] by placing tumor cells in mammary fat pad of female SCID mice (Fig. 6). Briefly, exponentially growing breast cancer cells (1×10^6) in 0.1 ml Hank's buffered salt solution (HBSS) were inoculated into the surgically exposed mammary fat pad (m.f.p.) of 7-8 weeks old female SCID mice. The growth of primary tumors was monitored weekly by measuring tumor diameters with calipers. Primary tumors were removed at 1cm of diameter (typically, 30-35 days after inoculation) and frozen in liquid N₂ or formalin-fixed. After 4-5 weeks, the mice were sacrificed and lungs, bones, livers, and lymph nodes were collected and analyzed histologically for metastases using the pathology core facility at RPCI. Mammary tumors were also evaluated by light microscopy and immunohistochemistry for expression of EGFP and HA-tagged Alk5 mutants. Twelve female SCID mice per group were used. Alk5-T204D reduced latency time for appearance of palpable tumors and enhanced growth of primary tumors without an effect on Ki67 proliferation index (Fig. 6).

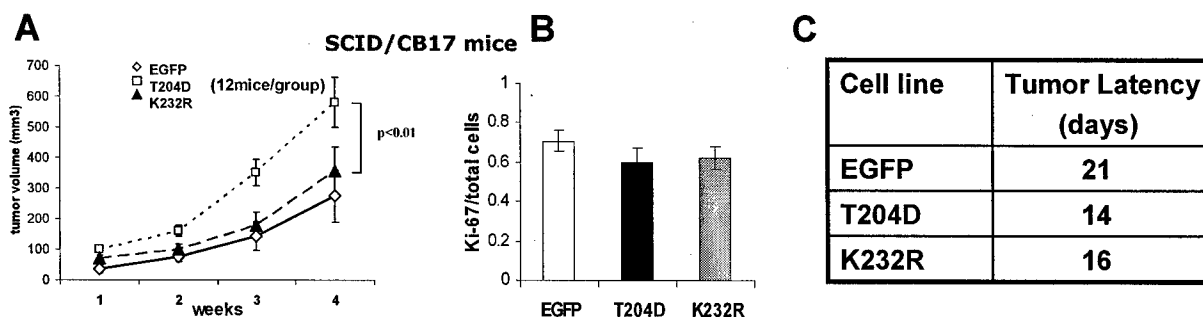


Fig. 6. (A) MDA-MB-231 cells expressing mutants of T β RI receptor and control EGFP cells were placed orthotopically at mouse mammary gland fat pad of 8-week old female SCID mice (1×10^6 /mice, 12 mice/group). (B) Proliferation index was determined as ratio of Ki67-positive cells relative to total cell numbers counted at 20X magnification. 5 fields per slide, at least 300 cells per field. (C) Tumor latency shows the time of appearance of palpable primary tumors. Tumor size was measured with calipers.

T204D cells formed significantly more spontaneous metastases to lungs compared to K232R and EGFP control cells (Fig. 7A). Examination of cell proliferation using MTS assay (Promega) showed that Alk5-T204D reduced cell proliferation by 40% compared to control EGFP-expressing cells, while Alk5-232R had not effect (Fig. 7B). Thus, the increase in macrometastases could not be explained by cell proliferation.

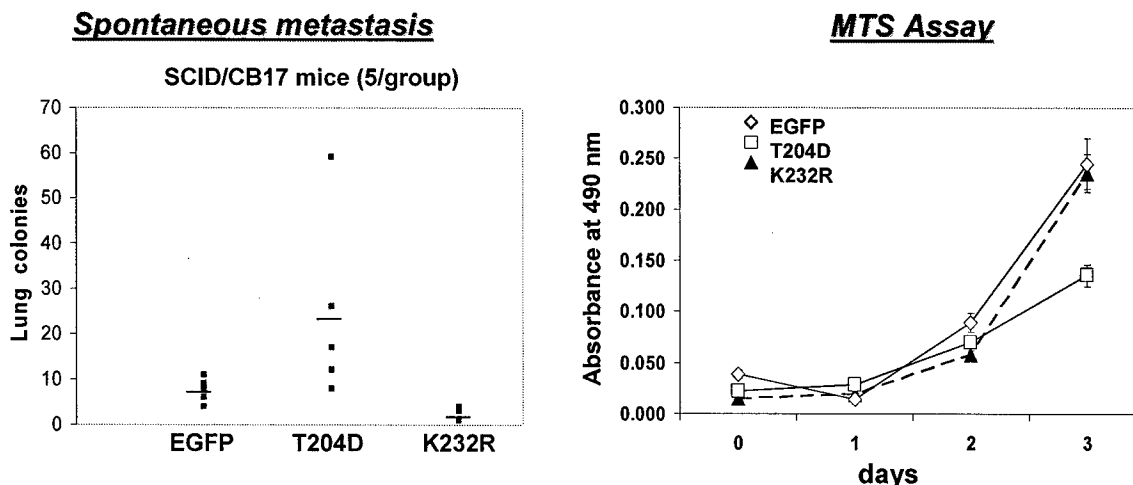


Fig. 7. (A) Xenografts of MDA-MB-231 cells expressing mutants of T β RI receptor and control EGFP cells were removed at 10 mm³ in size. Animals were euthanized and lungs were stained with 15% black Indian ink. Lung surface macro-metastases were counted. (B) Cell proliferation in culture was measured using MTS assay (Promega).

Matrigel invasion assay showed a 4-fold increase in invasion of matrigel-covered Boyden chambers by Alk5-T204D cells compared to EGFP control (Fig. 8). These results suggested that Alk5-T204D may increase activity of matrix proteinases.

To test this idea we examined activity of matrix metalloproteinases 2 and 9 (MMP2/9) using gelatin zymography assay (Fig. 9). Briefly, samples of the 48h-condition medium from tumor cells were resolved on 7.5% PAGE containing 0.1% gelatin. After re-naturation, gels were incubated for 24 h at 37°C in the reaction buffer containing 5mM CaCl₂. Gels were stained with Coomassie Blue R-250

and then digitized. The band densities were evaluated using NIH-Image software. The analysis showed that treatment with TGF- β resulted in a 1.8-fold increase in activity of MMP9 (gelatinase B, 92 kDa) in parental and EGFP-expressing cell lines without an effect on MMP2 (gelatinase A, 72kDa).

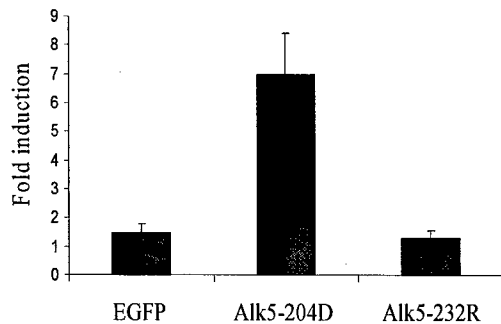


Fig. 8. Matrigel invasion assay showed a 4-fold increase in invasion of T β RI-T204D cells. Incubation time was 24 h, 1×10^5 cells loaded on membrane. Experiments were done in triplicate and cells were counted from five fields for each membrane.

Expression of kinase-inactive Alk5-K232R inhibited this response. Cells expressing kinase-active Alk5-T204D showed nearly 10-fold higher MMP9 activity compared to control EGFP cells independent of TGF- β . Treatment of cells with p38 MAPK inhibitor SB202190 reduced MMP9 activity by 90%, while HER2/ERBB2 specific inhibitor AG825 had no effect (Fig. 9). In control the ERBB2 inhibitor reduced cell proliferation by 90% (data not shown). ERBB2 has been shown to cooperate with Alk5 signaling in enhancing cell motility [5] and pulmonary metastasis [6].

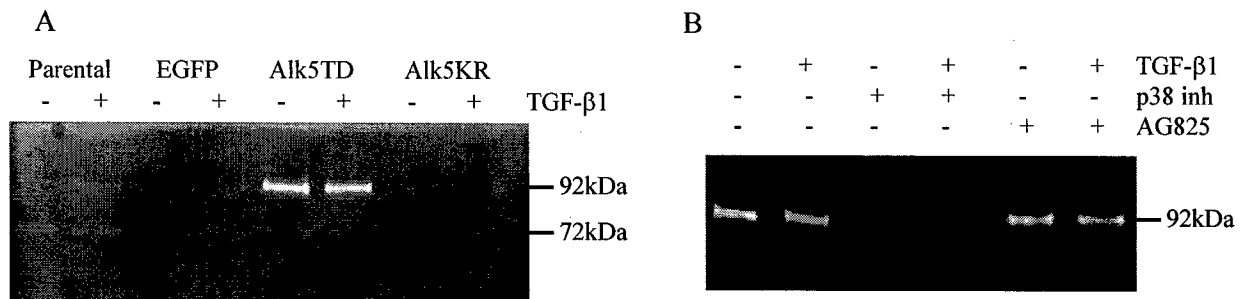


Fig. 9. Gelatin gel zymography assay in MDA-MB-231 cells. Where it is indicated cells were treated with 2ng/ml TGF- β 1 and 10 μ M p38 inhibitor, or 5 μ M AG825, a HER2/ERBB2 inhibitor.

These results suggest that p38 MAPK signaling is required for T β RI/Alk5-mediated increase in cell migration and invasion in part via activation of MMP9 in MDA-MB-231 cells. To address the role of MMP-9 in tumor cell migration, invasion, and metastasis we suppressed MMP-expression in MDA-MB-231 cells using RNA interference approach (Fig. 10).

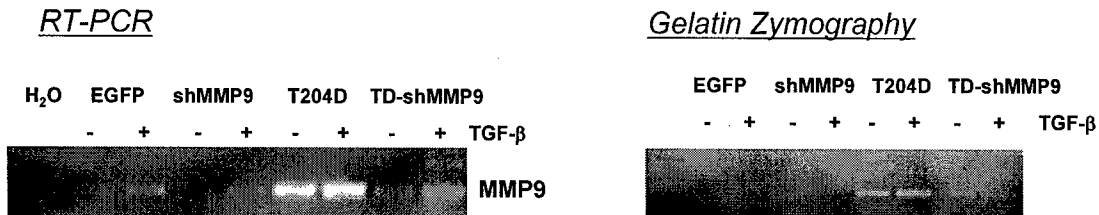


Fig. 10. (A) RT-PCR of MMP9 in MDA-MB-231 cells. Actin control showed equal loading. (B) Gelatin Zymography showed suppression of MMP9 activity in EGFP and T204D cells by interfering RNA to MMP9 (shMMP9). There were no off-target effects on MMP2, MMP14, or PAI-1 (data not shown).

Suppression of MMP9 decreased invasion of T204D cells examined in Matrigel invasion assay (Fig. 11A) but did not affect cell migration (data not shown). In experimental metastasis model, expression of RNAi to MMP9 (shMMP9) significantly reduced formation of lung metastases by

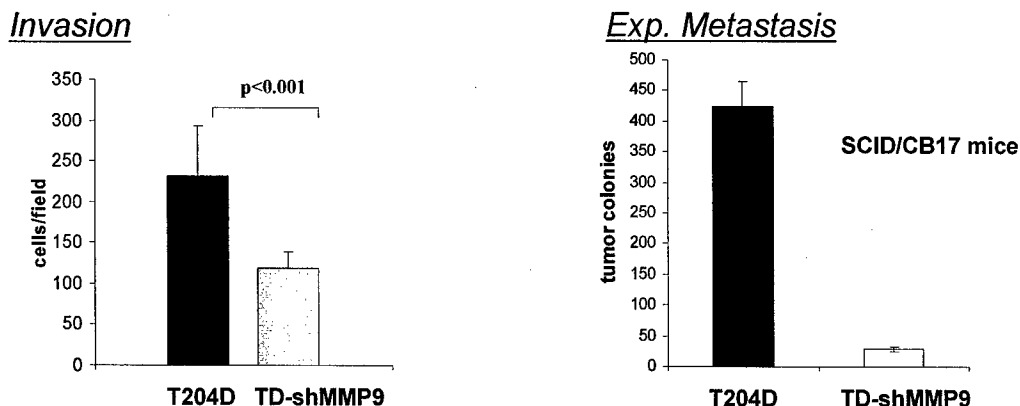


Fig. 11. (A) Suppression of MMP9 using RNAi in T β RI-T204D cells reduced invasion of Matrigel. Incubation time was 24 h, 1×10^5 cells loaded on membrane. Experiments were done in triplicate and cells were counted from five fields for each membrane. (B) MDA-MB-231 cells expressing active T β RI-T204D receptor alone or in combination with RNAi to MMP9 (TD-shMMP9) were injected in tail-vein of SCID mice (1×10^6 /mice, 8mice/group). Four weeks later mice were euthanized and lungs were stained with 15% black Indian ink. Lung surface macro-metastases were counted. $P < 0.001$ compared to control using ANOVA test.

T204D cells (Fig. 11B). shMMP9 did not affect cell proliferation suggesting that main effect is mediated by activity of MMP9 on tumor microenvironment.

RNAi to MMP9 (shMMP9) also significantly reduced latency of palpable tumor formation, xenograft growth, and formation of metastasis (Fig. 12). Since shMMP9 did not affect cell growth in culture we explore the possibility that shMMP9 affects tumor vascularization. Staining of tumor tissues showed a significant decrease of blood vessels within the tumor tissues in the presence of shMMP9 compared to T204D controls (Fig. 12B). These data further emphasize the idea that shMMP9 may play a role in TGF- β changes in tumor microenvironment.

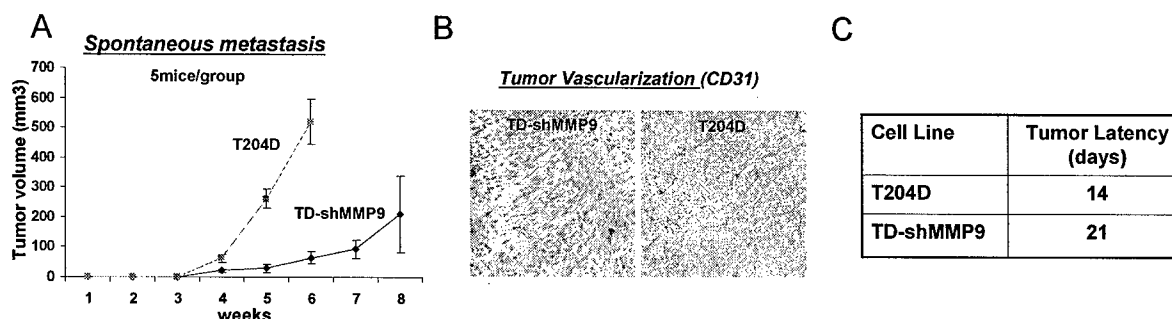


Fig. 12. (A) The growth of xenografts of MDA-MB-231 cells expressing (1) T204D or (2) T204D and shMMP9 was measured using calipers. (B) Immunohistochemical staining of tumor tissues using CD34 monoclonal antibody detecting endothelial cells. (C) Tumor latency shows the time of appearance of palpable primary tumors. Tumor size was measured with calipers.

To address the role of p38 MAPK signaling in metastasis we generated MDA-MB-231 cells expressing dominant negative mutants of kinases in the p38 pathway: non-tagged TAK1-K63W, HA-

tagged MKK6AL, and Flag-tagged p38AGF using retroviral vector pBMN-IRES-EGFP (Fig. 1). Preliminary results suggest that expression of dn-mutants did not reduce phosphorylation of Smad2/3 but dn-p38AGF decreased phosphorylation of downstream target HSP27 (Fig. 13).

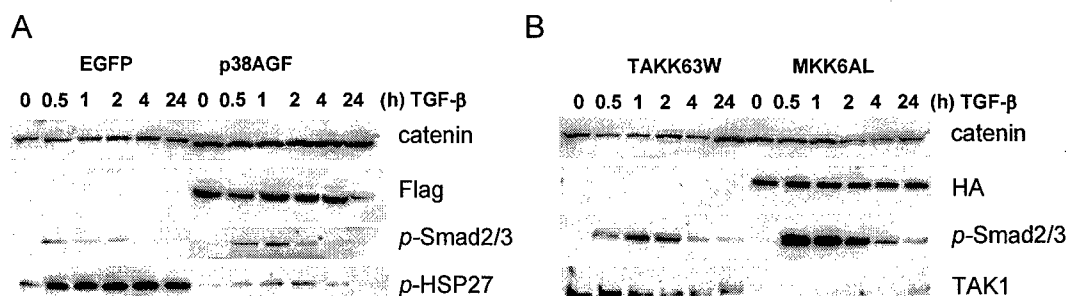


Fig. 13. (A-B) Immunoblot analyses of expression of kinase-mutants and their effects on phosphorylation of Smad2/3 and HSP27.

Examination of MMP9 activity in conditioned medium showed that dn-TAK1 and MKK6AL significantly reduced MMP9 gelatinase activity compared to EGFP cells (Fig. 14).

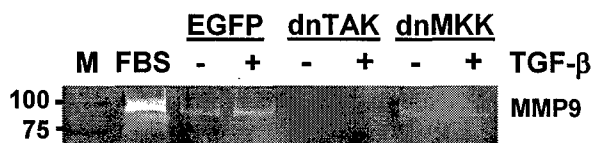


Fig.14. Gelatin Zymography showed reduction of MMP9 activity in dnTAK1 and dnMKK6 expressing cells compared to EGFP control. FBS (1/10 dilution) was used as a standard control. There were no effects on MMP2.

Further, cells expressing dn-mutants of TAK1 and p38 were examined in spontaneous and experimental metastasis studies (Fig. 15). Dn-mutants delayed appearance of palpable tumors and xenograft growth (Fig. 15 A-B). Dn-mutants also significantly reduced formation of lung surface metastases (Fig. 15C). The further investigation of these animal models is underway.

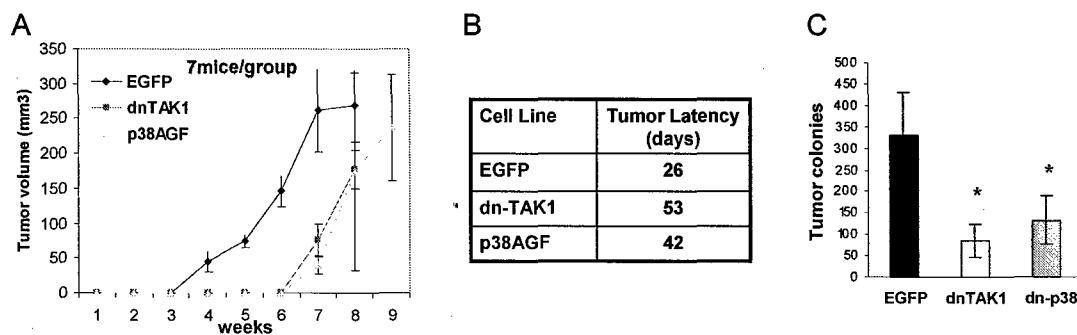


Fig. 15. (A) The growth of xenografts of MDA-MB-231 cells expressing EGFP control, dnTAK1-K63W, and dn-p38AGF was measured using calipers. (B) Tumor latency shows the time of appearance of palpable primary tumors. Tumor size was measured with calipers. (C) Experimental metastasis protocol. MDA-MB-231 cells were injected in tail-vein of SCID mice (1×10^6 /mice, 8mice/group). Four weeks later mice were euthanized and lungs were stained with 15% black Indian ink. Lung surface macro-metastases were counted. $P < 0.001$ compared to control using ANOVA test.

Thus, these studies suggest the p38MAPK pathway contribute in TGF- β -mediated metastasis by stimulating cell migration and invasion, as well by affecting tumor microenvironment via elevating expression of active MMP9.

Key Research Accomplishments

We found that:

- Constitutively active TGF- β type I receptor Alk5-T204D increased lung macro-metastases in spontaneous and experimental metastasis models in part by enhancing migration and invasion of tumor cells via the p38 MAPK pathway.
- T β RI-p38 MAPK signaling increased expression and activity of MMP9 but not MMP2. Alk5-T204D increased tumor cell invasion and MMP9 activity but reduced cell proliferation.
- Suppression of MMP9 with interfering RNA inhibited tumor growth and formation of metastases by MDA-MB-231 cells in SCID mice suggesting that MMP-9 is essential for T β RI-mediated breast cancer progression. Suppression of MMP9 inhibited invasion but not cell motility.
- Dominant negative TAK1 and MKK6 decreased expression of active MMP9 in MDA-MB-231 cells.
- Dominant negative TAK1 and p38 MAPK affected tumor latency and growth of MDA-MB-231 xenografts in SCID mice. MDA-MB-231 cells expressing dn-TAK1 or dn-p38 formed significantly reduced amount of lung surface metastases compared to control cells in experimental metastasis model in SCID mice.
- Together, these studies suggest that the T β RI-TAK1-MKK6-p38 MAPK signaling cascade contributes to cancer progression by stimulating cell migration and invasion, and by changing tumor microenvironment. MMP9 is a downstream mediator of this cascade that contributes in regulation of invasion and angiogenesis.

We developed *in vivo* tumor metastasis models using EGFP-expressing and genetically-modified MDA-MB-231 cells. These models will be used to address the following questions: (a) whether the T β RI-TAK1-MKK6-p38 MAPK signaling cascade is a potential target for anticancer/metastatic therapy; (b) whether the activity of this cascade has a prognostic value in breast cancer progression; (c) what is the mechanism of MMP9 regulation by the TAK1-MKK6-p38 MAPK pathway; (d) how MMP9 affects tumor angiogenesis. The intriguing possibility is regulation of VEGF secretion by MMP9.

Reportable Outcomes

Manuscripts:

1. **Bakin A.**, Rinehart, C., Tomlinson A. K., Arteaga C. L., p38 mitogen-activated protein kinase is required for TGF β -mediated fibroblastic transdifferentiation and cell migration. *J Cell Sci.* 2002 Aug 1;**115**(Pt 15):3193-206.
2. Dumont N., **Bakin A.V.**, and Arteaga CL. Autocrine TGF- β signaling mediates smad-independent motility in human cancer cells. *J Biol Chem* 2003 Jan 31;**278**(5):3275-85.
3. **Bakin AV**, Safina A, Rinehart C, Daroqui C, Darbary H, Helfman D. A critical role of tropomyosins in TGF-(beta) regulation of the actin cytoskeleton and cell motility in epithelial cells. *Mol Bio Cell*, 15:4682-4694, 2004.
4. **Bakin AV**, Stourman V, Sekhar RK, Rinehart C, Yan X, Meredith MJ, Arteaga CL, Freeman ML. Smad3-ATF3 signaling mediates TGF- β suppression of genes encoding phase ii detoxifying proteins. *Free Radical Biology and Medicine* 38:375-387, 2005.

Principal Investigator: Bakin, Andrei V. Award #DAMD17-02-1-0602

5. Varga AE, Stourman NV, Zheng Q, Safina A, Quan L, Li X, Sossey-Alaoui K, **Bakin AV**. Silencing of Tropomyosin-1 gene by DNA methylation alters tumor suppressor function of TGF- β Oncogene 1-10, 2005.
6. Rossi MR, Ionov Y, **Bakin AV**, Cowell JK. Truncating mutations in the ACVR2 gene attenuates activin signaling in prostate cancer cells. Cancer Genetics and Cytogenetics, In Press.
7. Safina A., Rinehart, C., Arteaga, C. L., **Bakin, A.V.** p38 MAPK, Sp1 and Smads contribute to TGF β -induced expression of fibronectin. (in preparation)
8. Safina A., Vendette E., **Bakin A.V.** p38 MAPK is required for Alk5-mediated tumor invasion and metastasis of breast cancer MDA-MB-231 cells. (in preparation)

Abstracts:

1. Bakin, A.V., Rinehart, C. TGF β regulates expression of target genes and p38 MAPK signaling to induce changes in the actin cytoskeleton. AACR, 2003, Abstract #102164.
2. Bakin, A.V., Rinehart, C., Arteaga, C. L. p38Mapk, Sp1 and Smads contribute to TGF β -induced expression of fibronectin. AACR Special Conference on the role of the TGFbeta superfamily in the pathogenesis of cancer and other diseases, January 15-19, 2003 La Jolla, CA
3. Bakin, A.V., Daroqui C., Safina A. TGF- β and ERK differentially regulate genes involved in the formation of stable actin filaments. Advances in Breast Cancer Research: Genetics, Biology, and Clinical Implications. October 8-12, 2003, Huntington Beach, CA.
4. Bakin, A.V., Daroqui C., Safina A. TGF- β and ERK signaling differentially regulate genes involved in actin stress fiber formation. Annual AACR meeting, 2004, Abstract # 130.
5. Bakin, A.V., Safina A., Daroqui C., Lei Q., Varga A., Helfman D., TGF- β regulation of epithelial cell motility via tropomyosin-mediated actin stress fibers. The 10th International Congress of the Metastasis Research Society "Progress Against Tumor Progresssion". September 17-20th, 2004. Genoa, Italy. Abstract #2 (Short talk).
6. Bakin AV, Varga A., Safina A., Zheng Q., The role of tropomyosin in metastatic switch in TGF-beta function during cancer progression. Annual AACR meeting, April 16-20th, 2005, Anaheim, CA. Abstract # 228.
7. Safina A., Vendette E., Varga A., Bakin AV, TGF-beta-p38MAPK signaling contributes to tumor invasion and pulmonary metastases by increasing MMP9 activity and cell motility. Annual AACR meeting, April 16-20th, 2005, Anaheim, CA. Abstract # 4347 (Minisymposium, Tumor Biology 30).

The following materials have been generated:

1. Retroviral vectors based on pBMN-IRES-EGFP, which encode: TGF- β type I receptor wild type, Alk5-WT, and kinase-inactive Alk5-232R, kinase-active Alk5-204D; dominant-negative (DN) mutants for p38 α , p38AGF; MKK3, MKK3AL; MKK6, MKK6AL; Rac1, RacN17; TGFbeta-activated kinase (TAK1), dominant-negative mutant TAK1-K63W; dominant-negative mutant PAK1-N205 (dn-PAK1).
2. MDA-MB-231 cell lines expressing wild type and mutants of Alk5 and TGF- β type II receptor; Rac1-N17; p38AGF; MKK6AL; dn-PAK1.
3. MDA-MB-231 expressing interfering RNA to MMP9.
4. MDA-MB-231 Tet-Off cell lines. In these cells, expression of a gene of interest is controlled by a tetracycline-regulated promoter.

Conclusions

Our studies suggest that the T β RI-TAK1-MKK6-p38 MAPK signaling cascade contributes to breast cancer progression by stimulating cell migration and invasion, and by changing tumor microenvironment. MMP9 is a downstream mediator of this cascade that significantly contributes to regulation of tumor invasion and angiogenesis. Thus, the TAK1-MKK6-p38 MAPK signaling cascade may represent a potential target for anticancer/metastatic therapy and the activity of this cascade may also serve as a marker of metastasis, and could be used for prognosis of breast cancer progression.

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Appendices

Biographical Sketch
Copies of 3 abstracts
Reprints of two papers

BIOGRAPHICAL SKETCH

Provide the following information for the key personnel and other significant contributors in the order listed on Form Page 2.
Follow this format for each person. **DO NOT EXCEED FOUR PAGES.**

NAME Andrei V. Bakin		POSITION TITLE Assistant Professor	
eRA COMMONS USER NAME			
EDUCATION/TRAINING <i>(Begin with baccalaureate or other initial professional education, such as nursing, and include postdoctoral training.)</i>			
INSTITUTION AND LOCATION	DEGREE <i>(if applicable)</i>	YEAR(s)	FIELD OF STUDY
Moscow State University, Moscow, Russia	M.S.	1984	Chemistry/ Biochemistry
Moscow State University, Moscow, Russia	Ph.D.	1990	Molecular Biology

A. POSITIONS AND HONORS

1990-1991 Research Fellow, A. N. Belozersky Institute of Physico-Chemical Biology, Moscow State University, Moscow USSR

1990-1991 Visiting Scientist, Department of Chemistry, University of Texas, Austin, TX

1991-1995 Postdoctoral Fellow, Roche Institute of Molecular Biology, Nutley, NJ

1995-1999 Postdoctoral Fellow, Department of Developmental Neurobiology, St. Jude Children's Research Hospital, Memphis, TN

1999-2003 Research Assistant Professor, Dept. of Medicine, Vanderbilt University, Nashville, TN

2003-present Assistant Professor, Dept. of Cancer Genetics, Roswell Park Cancer Institute, Buffalo, NY

B. PUBLICATIONS (Selected from 27 peer reviewed articles)

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C. RESEARCH SUPPORT

ACTIVE

P.I. – Andrei Bakin 2/15/2002 – 1/31/2007
National Institutes of Health
R01CA095263
Mechanisms of TGFbeta-mediated fibroblastic transition

The project tests the hypothesis that activation of the p38Mapk pathway by kinase-active TGF- β receptors mediated by Rho-family GTPases is required for setting up a program of changes in the cell phenotype leading to TGF β -induced fibroblastic transition and cell migration.

P.I. – Andrei Bakin
Department of Defense
DAMD17-02-01-0602

p38 mitogen-activated protein kinase in metastasis associated with transforming growth factor beta

The major goal of this project is to examine the role of TGFbeta-activated kinase 1 (Tak1) and Pak1 in activation of p38Mapk pathway in metastasis associated with TGFβ1.

Abstract #4347

Title: TGF-beta-p38MAPK signaling contributes to tumor invasion and pulmonary metastases by increasing MMP9 activity and cell motility

Authors: Alfiya Safina, Erika Vandette, Andrea Varga, Andrei V. Bakin,

Affiliation: Roswell Park Cancer Institute

Transforming growth factor beta (TGF-b) is a potent tumor suppressor but at the late stages of cancer it can promote formation of highly metastatic tumors by stimulating epithelial to mesenchymal transition (EMT), cell migration, and changes in tumor microenvironment. We have shown that the p38 mitogen activated protein kinase (p38MAPK) pathway contributes in TGF-b-mediated EMT and tumor cell migration (Bakin et al., JCS, 2002). In this study we investigated the contribution of p38MAPK signaling in TGF-beta-mediated metastasis of breast cancer MDA-MB-231 in spontaneous and experimental metastasis mouse models. Expression of constitutively active TGF-b type I receptor Alk5-204D in MDA-MB-231 (MB-231) cells enhanced cell migration, whereas kinase-inactive receptor Alk5-232R reduced cell migration. Alk5-204D increased by 4-fold tumor cell invasion in the matrigel invasion assay. Examination of matrix metalloproteinase activity in zymography assays showed an 8-fold increase in MMP9 activity but no effect on MMP2 and MMP1. Application of p38MAPK inhibitors reduced MMP9 activity and blocked cell invasion. RT-PCR showed a 4-fold increase in MMP9 mRNA in Alk5-204D cells compared to control cells. Alk5-204D-expressing cells formed 4-times more pulmonary macrometastatic lesions compared to control cells when injected in tail vein of SCID mice ($P < 0.01$). To study TGF-b effects on the spontaneous metastasis, tumor cells were placed into mammary fat pad of SCID female mice. Alk5-204D significantly reduced tumor latency and enhanced primary tumor growth compared to control and Alk5-232R cells. Analysis of metastasis showed that Alk5-204D cells formed significantly more pulmonary metastases compared to control and Alk5-232R cells. To investigate the role of p38MAPK in metastasis, we generated cell lines expressing dominant negative (dn) MKK6AL and p38AGF. MMP9 in MDA-MB-231 and Alk5-204D cells was suppressed using shRNA to human MMP9. DN-mutants reduced cell motility, while shMMP9 reduced MMP9 activity examined in zymography assays. The effect of the dn-mutants and shMMP9 on metastasis is under investigation. These studies suggest that TGF-b signaling enhances tumor cell invasion and metastasis by increasing MMP9 activity and cell migration via p38MAPK dependent mechanisms.

This work was supported by DOD grant DAMD17-02-1-0602.

Abstract #228

The role of tropomyosin in metastatic switch in TGF-beta function during cancer progression

Authors: Andrei V. Bakin, Qiao Zheng, Alfiya Safina, Andrea Varga

Affiliation: Roswell Park Cancer Institute

Transforming growth factor beta (TGF-b) functions as a tumor suppressor by controlling cell growth, differentiation, and apoptosis. However, at late stages of tumorigenesis it contributes to metastasis by inducing mesenchymal-like phenotype in tumor cells and promoting cell motility and invasion. The mechanisms underlying the metastatic switch in TGF-b function are not well understood. We have recently reported that epithelial to mesenchymal transition (EMT) is associated with TGF-b induction of high molecular weight tropomyosins (HMW TMs) that leads to formation of stable actin microfilaments or stress fibers. Actin microfilaments are essential cytoskeletal structures involved in cell morphology and motility. Surprisingly, induction of stress fibers by TGF-b inversely correlated with metastatic behavior of tumor cells. Here, we investigated the mechanisms underlying loss of stress fiber formation in response to TGF-b in metastatic cells and the role of tropomyosin-mediated stress fibers in TGF-b control of cell migration. Tropomyosin is a dimeric coil-coiled protein that binds along actin microfilaments forming a head-to-tail polymer in the actin helical groove. TMs stabilize actin microfilaments and protect them from de-polymerizing proteins like gelsolin and cofilin. We analyzed a panel of normal epithelial (NMuMG, MCF10A) and carcinoma (MCF7, A549, MDA-MB-231, SW620, SW480) cell lines for expression of HMW TMs encoded by TPM1 and TPM2 genes. Both genes were expressed in normal and non-metastatic tumor cell lines. However, in breast and colon metastatic tumor cell lines, TPM1 was significantly reduced or was absent, whereas TPM2 was expressed at low levels. Reduced expression of tropomyosin correlated with high activity of Ras-MEK signaling. Inhibition of the Ras-MEK pathway in MDA-MB-231 cells by the MEK inhibitor U0126 or by the dominant negative mutant MEK1 markedly increased TGF-b-induced expression of tropomyosin, enhanced stress fibers and blocked cell migration. Retrovirus-mediated expression of oncogenic H-RasV12 in A549, MCF7 and NMuMG cells significantly reduced tropomyosin resulting in the loss of stress fibers and enhancement of cell motility and invasion. We generated MDA-MB-231-TetOff-TM3 cell line in which expression of tropomyosin 3 isoform (a TPM1 gene product) was regulated by doxycycline. Withdrawal of doxycycline resulted in a 5-fold induction of TM3 that was nearly 50% of the tropomyosin level in MCF7. Concurrent with induction of TM3, cell migration was inhibited by 45%. Together these data suggest that tropomyosin-mediated stress fibers have an essential role in TGF-b control of cell motility and invasion, and the loss of this TGF-b response is a critical step in the acquisition of metastatic phenotype by tumor cells.

This work was supported in part by PHS grant R01 CA62212 and DOD grant DAMD17-02-1-0602.

The 10th International Congress of the Metastasis Research Society "Progress Against Tumor Progression". September 17-20th, 2004. Genoa, Italy. Abstract #2 (Short talk).

Abstract #2

TGF- β regulation of epithelial cell motility via tropomyosin-mediated actin stress fibers

Andrei V. Bakin, Alfiya Safina, Cecilia Daroqui, Quan Lei, Andrea Varga, and David Helfman

Transforming growth factor beta (TGF- β) signaling pathway is a major cellular growth inhibitory and pro-apoptotic pathway in epithelial, endothelial, hematopoietic, and other cell types. Paradoxically, TGF- β has been implicated in promotion of the metastatic behavior of tumor cells by inducing epithelial to mesenchymal transition (EMT), tumor cell migration and invasion. We identified a novel pathway contributing to TGF- β control of cell motility in epithelial cells. We show that tropomyosins induced by TGF- β via Smad and p38Mapk signaling mediate stress fiber formation and control of cell motility. Silencing of tropomyosins with short interfering RNAs (siRNAs) blocks stress fiber assembly while ectopic expression of tropomyosins results in stress fibers. Ectopic-expression and siRNA experiments show that Smad3 and Smad4 mediate up-regulation of tropomyosins and stress fiber formation. Chromatin immunoprecipitation experiments demonstrate that Smad3/4 bind to the TPM1 promoter in response to TGF- β . TGF- β induction of tropomyosins and stress fibers are suppressed by Ras-ERK signaling in metastatic breast cancer cell lines. Inhibition of Ras-ERK signaling restores TGF- β regulation of tropomyosins, stress fibers, and reduces cell motility. These results suggest that ERK signaling may inhibit TGF- β induction of stress fibers by suppressing Smad-dependent expression of tropomyosins. The suppression of tropomyosins by oncogenic pathways including Src and Ras can represent a common route to altering TGF- β control of cell motility and contribute to the acquisition of metastatic phenotype by tumor cells. Thus, restoration of TGF- β stress fiber response represents a novel target for the development of effective antimetastatic therapies and tropomyosins are potential metastasis suppressors.

This work was supported in part by PHS grant R01 CA62212 and DOD grant DAMD17-02-1-0602.

A Critical Role of Tropomyosins in TGF- β Regulation of the Actin Cytoskeleton and Cell Motility in Epithelial Cells

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Monitoring Editor: Thomas Pollard

We have investigated transforming growth factor beta (TGF- β)-mediated induction of actin stress fibers in normal and metastatic epithelial cells. We found that stress fiber formation requires de novo protein synthesis, p38Mapk and Smad signaling. We show that TGF- β via Smad and p38Mapk up-regulates expression of actin-binding proteins including high-molecular-weight tropomyosins, α -actinin and calponin h2. We demonstrate that, among these proteins, tropomyosins are both necessary and sufficient for TGF- β induction of stress fibers. Silencing of tropomyosins with short interfering RNAs (siRNAs) blocks stress fiber assembly, whereas ectopic expression of tropomyosins results in stress fibers. Ectopic-expression and siRNA experiments show that Smads mediate induction of tropomyosins and stress fibers. Interestingly, TGF- β induction of stress fibers was not accompanied by changes in the levels of cofilin phosphorylation. TGF- β induction of tropomyosins and stress fibers are significantly inhibited by Ras-ERK signaling in metastatic breast cancer cells. Inhibition of the Ras-ERK pathway restores TGF- β induction of tropomyosins and stress fibers and thereby reduces cell motility. These results suggest that induction of tropomyosins and stress fibers play an essential role in TGF- β control of cell motility, and the loss of this TGF- β response is a critical step in the acquisition of metastatic phenotype by tumor cells.

INTRODUCTION

There is solid evidence that the transforming growth factor beta (TGF- β) signaling pathway is a major cellular growth inhibitory and proapoptotic pathway in epithelial, endothelial, hematopoietic, and other cell types (Roberts and Wakefield, 2003). However, clinical and experimental studies indicate that metastatic cancers of the breast and other tissues express elevated levels of TGF- β that appears to support the metastatic behavior of the tumor cells (Saito *et al.*, 2000; Derynck *et al.*, 2001). This apparent paradox has been associated with a progressive decline in the antitumorigenic function and a gain of protumorigenic activities of TGF- β , including induction of epithelial to mesenchymal transition (EMT) and tumor cell migration and invasion (Derynck *et al.*, 2001; Wakefield and Roberts, 2002). Oncogenic Ras, Src, and

ErbB2 as well as alterations in TGF- β signaling mediated by Smads, mitogen-activated protein kinases (Mapks), Rho kinases, and Akt/PKB are thought to contribute to the metastatic phenotype (Derynck and Zhang, 2003; Roberts and Wakefield, 2003).

The actin cytoskeleton plays a central role in the regulation of cellular processes linked to metastasis including cell proliferation, apoptosis, anchorage-independent cell growth, and cell migration and invasion (Pawlak and Helfman, 2001; Jaffe and Hall, 2002). TGF- β induces a rapid reorganization of the actin cytoskeleton, leading to membrane ruffling at the cell edges in both nontumorigenic and tumorigenic epithelial cells, whereas a prolonged incubation with TGF- β results in the formation of stress fibers (Bakin *et al.*, 2002; Edlund *et al.*, 2002). The immediate TGF- β -mediated changes in the actin cytoskeleton have been associated with activation of the Rho family of GTPases, Rac, CDC42, and RhoA (Bakin *et al.*, 2002; Edlund *et al.*, 2002), which control cell motility and invasive phenotypes by regulating organization of actin filaments (Jaffe and Hall, 2002). TGF- β regulates activity of these GTPases in various epithelial cell lines independently of Smad signaling (Bhowmick *et al.*, 2001; Bakin *et al.*, 2002; Edlund *et al.*, 2002). The interplay between Rho-like GTPases regulate both the protrusive and contractile forces required for cell migration, via a combination of actin polymerization, depolymerization, and the interaction of myosin-based motors with actin filaments (Etienne-Manneville and Hall, 2002). Although RhoA contributes to cell migration by inducing actomyosin contractility, RhoA can also inhibit cell movement by stimulating the assembly of stress fibers and focal adhesions associated with the cell substratum (Cox *et al.*, 2001). The TGF- β

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Abbreviations used: TGF- β , transforming growth factor beta; Mapk, mitogen-activated protein kinase; TM, tropomyosin; siRNA, short-interfering RNA. NMuMG cells that were untreated or treated with 2 ng/ml TGF- β 1 for 24 h in the absence or presence of 10 μ M SB202190. The data represent an average of three independent experiments. Actn1, α -actinin1; Tpm3, tropomyosin3; Tpm4, tropomyosin4; Cnn2 (H2), calponin2 (H2).

induction of actin stress fibers has been shown to depend on Smad signaling (Piek *et al.*, 1999b), the RhoA-Rho kinase pathway (Bhowmick *et al.*, 2001), and p38Mapk signaling (Hannigan *et al.*, 1998; Bakin *et al.*, 2002; Edlund *et al.*, 2002). However, the cellular targets regulated by these pathways and their roles in TGF- β regulation of stress fibers and cell motility have not been defined.

Oncogenic transformation mediated by Ras and Src results in the disruption of actin stress fibers and focal adhesions, whereas restoration of actin stress fibers inhibits cell transformation and reduces metastasis (Pawlak and Helfman, 2001). The mechanisms mediating the disruption of stress fibers by the Ras-ERK pathway involve inhibition of the RhoA/ROCK pathway (Sahai *et al.*, 2001; Pawlak and Helfman, 2002a, 2002b; Vial *et al.*, 2003) and repression of actin-binding proteins involved in stabilization of actin filaments including tropomyosins and α -actinin (Pawlak and Helfman, 2001). Thus, the Ras-Erk pathway may modify TGF- β regulation of stress fibers and cell motility through one or both of these mechanisms.

In this study we demonstrate that expression of tropomyosins mediated by Smad and p38Mapk signaling is required for TGF- β regulation of stress fibers and cell motility. We show that the Ras-ERK pathway antagonizes TGF- β induction of stress fibers by suppressing expression of tropomyosins. TGF- β does not modulate cofilin phosphorylation, suggesting that the RhoA-ROCK-LIM kinase-cofilin pathway is not rate limiting. We provide evidence that tropomyosins are both necessary and sufficient for TGF- β induction of stress fibers. We show that expression of tropomyosins in metastatic cells results in stress fibers and reduces cell motility. These results suggest that loss of TGF- β -induced stress fibers is an essential characteristic of a prometastatic conversion of TGF- β function and that regulation of tropomyosin expression is an important component of this response.

MATERIALS AND METHODS

Antibodies, Plasmids, and Other Reagents

TGF- β 1 was obtained from R&D Systems (Minneapolis, MN). The following antibodies were obtained from: to Smad2/3 (BD Transduction Laboratories, BD Biosciences, Lexington, KY); rabbit polyclonal to hemagglutinin (HA) epitope (Santa Cruz Biotechnology, Santa Cruz, CA); mouse monoclonal antibodies to tropomyosin (TM311), α -actinin, actin, and the Flag epitope (Sigma, St. Louis, MO); to phospho-Smad2, phospho-ERK1/2, phospho-p38Mapk, phospho-ATF2, phospho-HSP27, and HSP27 (Cell Signaling Technology, Beverly, MA). Phalloidin-Alexa Green and phalloidin-Texas Red were from Molecular Probes (Eugene, OR). Fluorescein-labeled anti-HA antibody was from Roche Applied Science (Indianapolis, IN). Plasmids encoding rat HA-tagged TM2 and TM3 isoforms were described previously (Gimona *et al.*, 1995). Inhibitors of p38Mapk (SB202190, SB203580, PD165319), MEK1/2 (PD098059, U0126), JNK (SP600125), Raf kinase, and protein synthesis (cycloheximide) were from Calbiochem (La Jolla, CA). Phospho-cofilin antibody was provided by Dr. James Bamburg, Colorado State University (Fort Collins, CO).

Cell Culture

Mouse mammary epithelial NMuMG cells, human cervical carcinoma SiHa cells, human breast cancer MDA-MB-231 cells, human epidermoid carcinoma A431 cells, and human kidney HEK293T cells were purchased from American Tissue Culture Collection (ATCC, Manassas, VA). Cells were cultured as recommended by ATCC.

Adenoviral Infection of Cells

Adenoviruses encoding EGFP, Flag-tagged Smads, and the HA-tagged constitutively active TGF- β type I Alk5T204D receptor were produced using HEK-293T cells and stored in aliquots at -80°C . Cells grown on plastic dishes or glass coverslips were incubated for 3 h with supernatant containing adenoviruses at 5–10 MOI. Medium was replenished and cells were grown for additional 24 h before further treatments.

RNA Isolation and cDNA Microarray Analysis

RNA from mouse nontumor mammary epithelial NMuMG cells treated with 2 ng/ml TGF- β 1 for 8 and 24 h was extracted as described in Bakin and Curran, (1999). Total RNA from each sample (35 μg) was labeled and hybridized with the NIA 15K Mouse array. Detailed descriptions of labeling and hybridization procedures are available from <http://array.mc.vanderbilt.edu/Pages/Protocols/Protocols.htm>. The array slides were scanned with an Axon 4000 scanner (Axon Instruments, Foster City, CA) at a resolution of 10 μm . The reference RNA from untreated NMuMG cells was labeled by using cyanine 3-dUTP (Cy3), and the RNA samples from TGF β 1-treated cells were labeled with cyanine 5-dUTP (Cy5). This experiment was performed in triplicate. Data were analyzed using GenePix4.0 software.

Immunoblot Analysis

Cells were incubated in medium containing 5% serum for 24 h before treatment with 2 ng/ml TGF- β 1. Cells were lysed in buffer containing 20 mM Tris, pH 7.4, 137 mM NaCl, 1% NP-40, 10% glycerol, 20 mM NaF, 1 mM Na orthovanadate, 1 mM PMSF, 2 $\mu\text{g}/\text{ml}$ aprotinin, and 2 $\mu\text{g}/\text{ml}$ leupeptin. Immunoblot analyses of protein extracts were performed as described (Bakin *et al.*, 2002).

Northern Blot and Reverse Transcription-Polymerase Chain Reaction Analysis

A cDNA fragment of rat TM3 and a polymerase chain reaction (PCR)-generated fragment of α -actinin1 cDNA spotted on the microarrays (GenBank accession number BG077689) were used as probes for Northern blot analysis. Identity of α -actinin1 and calponin2 cDNAs were verified by DNA sequencing matched through BLAST analysis (www.ncbi.nlm.nih.gov/BLAST/). Total RNA samples (15 $\mu\text{g}/\text{lane}$) obtained from NMuMG cells treated with 2 ng/ml TGF- β 1 for 4, 8, and 24 h were subjected to Northern blot analysis as described previously (Bakin and Curran, 1999). Amplification of transcripts was performed using 50 ng/ μL of total RNA and one-step reverse transcription (RT)-PCR system from Invitrogen (Carlsbad, CA) according to the manufacturing protocol. Primer sequences: β -actin, accession no. NM_007393, forward: GCTGGTCGTCGACAACGGCTC, reverse: CAAACATGATCTGGGTCATCTTTTC; α -tropomyosin, accession no. NM_024427.2, forward: GCTGGTGCACTGCAAAAGA, reverse: CCTGAGCTCCAGTGACTTC; β -tropomyosin, accession no. NM_009416.2, forward: AAGGATGCCAGGAGAAACT, reverse: CTTCCTTCAGCTGCATCTCC; calponin2, accession no. NM_007725.1, forward: ACCCTGTGGACCTGTTTAC, reverse: TGGAAGAGTTGTCCGACTTG; PAI-1, accession no. M33960.1, forward: CCACCGACTTCGGAGTAAAA, reverse: GCGTGTCAGCTCGTCTACAG.

Immunofluorescence Microscopy

Cells (10^5 cells/well) were grown in DMEM containing 5% fetal bovine serum (FBS) on glass coverslips (22×22 mm) for 24 h before treatment with 2 ng/ml TGF- β 1. Cells were fixed with 4% paraformaldehyde and stained as described (Bakin *et al.*, 2002). Actin filaments (F-actin) were stained with phalloidin-Alexa Green or phalloidin-Texas Red, and tropomyosins were visualized using TM311 antibody. Fluorescent images were captured using Zeiss Axiophot upright microscope (Thornwood, NY) and Nikon TE2000-E inverted microscope (Garden City, NY). In some experiments cells were permeabilized with 0.05% Triton X-100 for 10 min followed by fixation and staining.

Wound Closure Assay

The assay was performed as described previously (Bakin *et al.*, 2002). MDA-MB-231 cells ($1-2 \times 10^5$ /well) were seeded in 12-well plates and incubated in serum-free IMEM (Invitrogen) medium for 24 h before wounding with plastic tip across the cell monolayer. Kinase inhibitors were added 1 h before wounding. The cells were left untreated or treated with 2 ng/ml TGF- β 1 for 16 h. The wound closure was estimated as the ratio of the remaining wounded area relative to the initial area. Experiments were repeated at least three times.

Transcriptional Assay

NMuMG cells (3×10^4) were seeded in 24-well plates and transfected with 0.16 $\mu\text{g}/\text{ml}$ pSBE-Lux containing 12 repeats of Smad binding sequence (provided by J.-M. Gauthier, Laboratoire Glaxo Wellcome, Les Ulis Cedex, France) with 0.002 $\mu\text{g}/\text{ml}$ pCMV-Rl (Promega, Madison, WI) using FuGENE6 reagent (Roche Molecular Biochemicals, Indianapolis, IN) according to the manufacturer's protocol. Cells were incubated for 8 h in 0.5% FBS-DMEM before treatment with 1 ng/ml TGF- β 1 for 16 h. Firefly luciferase (Luc) and *Renilla reniformis* luciferase (Rl) activities in cell lysates were determined using the Dual Luciferase Reporter Assay System (Promega) according to the manufacturer's protocol in a Monolight 2010 luminometer (Analytical Luminescence Laboratory, San Diego, CA). Luc activity was normalized to Rl activity and presented as Relative Luciferase Units. All assays were done in triplicate wells and each experiment was repeated at least twice.

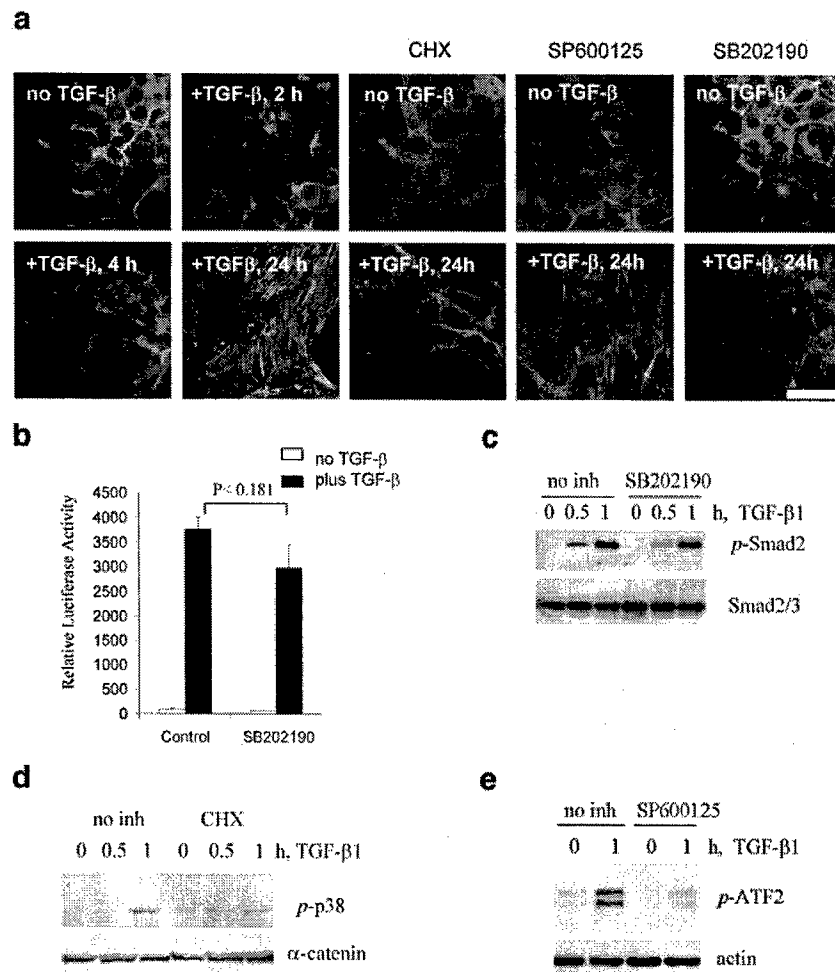


Figure 1. TGF- β 1-induced actin stress fibers require p38Mapk and a novel protein synthesis. (a) Actin filaments staining with phalloidin-Alexa Green in cells treated with 2 ng/ml TGF- β 1 in the absence or presence of inhibitors. Where it is indicated, cells were treated with 10 μ M cycloheximide (CHX) starting at 6 h after addition of TGF- β 1. Kinase inhibitors (15 μ M SP600125, a JNK inhibitor, and 10 μ M SB202190, a p38Mapk inhibitor) were added 1 h before addition of TGF- β 1. Scale bar, 15 μ m. (b) Luciferase activity in NMuMG transfected with Smad-dependent reporter pSBE-Lux and pCMV-Rl vectors and treated with 1 ng/ml TGF- β 1 for 16 h in the absence or presence of 15 μ M SB202190. Each bar represents the mean \pm SD of three wells. P value was determined by *t* test. The difference in luciferase activity in control and SB202190-treated cells is not statistically significant. (c) Immunoblot analysis of Smad2 phosphorylation in protein extracts (35 μ g/well) from NMuMG cells treated with 2 ng/ml TGF- β 1 in the absence or presence of 10 μ M SB202190. (d) p38Mapk phosphorylation in response to TGF- β 1 in protein extracts (35 μ g/well) from SiHa cells cotreated with 10 μ g/ml cycloheximide (CHX). (e) Inhibition of ATF2 phosphorylation by JNK inhibitors in protein extracts (35 μ g/well) from NMuMG cells treated with 2 ng/ml TGF- β 1.

Short Interference RNA Studies

RNA duplexes against human (cat. no. M-003902) and mouse (cat. no. M-004199) Smad4 were obtained from Dharmacon Research, Inc. (Lafayette, CO). RNA duplexes against tropomyosin (target sequence: AAGCAGCTG-GAAGATGAGC) were designed using the siDESIGN program at the Dharmacon siDESIGN center. A scramble control RNA duplex labeled with rhodamine was obtained from Qiagen (Chatsworth, CA). Cells were transfected with RNA duplexes using Oligofectamine reagents (Invitrogen) following the manufacturers protocol. The cells were transferred onto glass coverslips or plastic dishes. Forty-eight hours posttransfection, the cells were treated with TGF- β 1 for 24 h followed by immunoblot and immunofluorescence analysis.

RESULTS

TGF- β -induced Actin Stress Fiber Formation in Epithelial Cells Requires De Novo Protein Synthesis and p38Mapk

The mechanism of TGF- β -induced stress fiber (SF) formation was characterized in NMuMG mouse mammary epithelial cells. These cells exhibit a cuboidal cell morphology and a cortical organization of actin filaments in adhesion belts. Treatment of the cells with TGF- β 1 for 24 h induced formation of actin microfilament bundles (Figure 1). Actin filaments in adhesion belts were not significantly affected in the first 4 h of TGF- β treatment compared with untreated cells (Figure 1a), and SFs were not observed until 8 h after TGF- β addition. SFs were well developed in cells incubated with TGF- β for 24 h. This TGF- β response was blocked by treatment of cells with the p38Mapk inhibitor, SB202190,

suggesting involvement of p38Mapk in TGF- β -regulated SFs. This inhibitor did not significantly affect phosphorylation of Smad2 and TGF- β -mediated activation of the Smad-dependent luciferase reporter activity (Figure 1, b and c). Similar results were also obtained with two other p38Mapk inhibitors (SB203580 and PD165319; our unpublished results). Treatment of cells with the JNK inhibitor, SP600125, did not block TGF- β -mediated SF formation (Figure 1a), although it effectively blocked phosphorylation of ATF2 (Figure 1e). Expression of kinase-inactive mutant of mitogen-activated protein kinase kinase 6 (MKK6) blocked phosphorylation of p38Mapk and SF formation (Bakin *et al.*, 2000, 2002). In previous studies we have shown that TGF- β does not activate the ERK pathway in NMuMG cells and inhibition of MEK1/2 does not block stress fiber formation. Similar results were obtained for human cervical carcinoma SiHa cells, in which TGF- β induces p38Mapk signaling and SFs (Bakin *et al.*, 2002). To investigate whether this process depends on de novo protein synthesis, cells were treated with cycloheximide, the protein synthesis inhibitor. Cycloheximide blocked SF formation when added as late as 6 h after initiation of TGF- β treatment without inhibition of p38Mapk activation (Figure 1, a and d), but it had no effect after 12 h (our unpublished results). Thus, de novo protein synthesis and p38Mapk activity are required for TGF- β -mediated actin SF formation in epithelial cells.

Table 1. Regulation of genes encoding actin-binding proteins by TGF- β 1 in NMuMG cells

Gene	Accession no. of cDNA clones	Fold Change \pm SE	
		TGF- β 1	TGF- β 1 + SB202190
1 Actn1	BG077689	3.03 \pm 0.66	2.20 \pm 0.18
2 Actn1	BG065930	2.94 \pm 0.48	2.37 \pm 0.48
3 α -Tropomyosin	BG086016	1.81 \pm 0.39	1.48 \pm 0.09
4 α -Tropomyosin	BG079039	2.15 \pm 0.57	1.30 \pm 0.04
5 β -Tropomyosin	BG076419	2.48 \pm 0.09	1.52 \pm 0.20
6 β -Tropomyosin	BG073088	2.37 \pm 0.36	1.37 \pm 0.36
7 β -Tropomyosin	BG087093	2.83 \pm 0.43	1.83 \pm 0.48
8 Tpm3	BG077822	1.28 \pm 0.12	1.38 \pm 0.19
9 Tpm3	BG078017	1.22 \pm 0.08	1.40 \pm 0.08
10 Tpm3	BG064681	1.28 \pm 0.07	1.40 \pm 0.08
11 Tpm3	BC029186	1.16 \pm 0.15	0.91 \pm 0.11
12 Tpm4	BG077934	0.96 \pm 0.11	0.90 \pm 0.18
13 Tpm4	AW537534	1.34 \pm 0.12	1.46 \pm 0.22
14 Cnn2 (H2)	BG079442	3.00 \pm 0.25	3.11 \pm 0.29

cDNA microarray analysis was performed using total RNA from NMuMG cells, which were untreated or treated with 2 ng/ml TGF- β 1 for 24 h in the absence or presence of 10 μ M SB202190. The data represent an average of three independent experiments.

TGF- β Up-regulates Expression of Genes Encoding Actin-binding Proteins

To identify TGF- β target genes that mediate actin remodeling, we compared gene expression profiles in NMuMG cells before and after treatment with TGF- β 1 for 24 h

using mouse cDNA microarrays. The results indicate that expression of 62 genes changed more than twofold after treatment with TGF- β 1. Among these genes TGF- β stimulated expression of several genes encoding actin-binding proteins including tropomyosins (TM), α -actinin1, and calponin2 (Table 1). These proteins are known to be involved in the assembly of stable actin microfilament bundles (Ayscough, 1998; Danninger and Gimona, 2000; Tseng *et al.*, 2002; Hossain *et al.*, 2003). The α -tropomyosin and β -tropomyosin genes encoding high-molecular-weight tropomyosins were up-regulated 2–2.6-fold and were represented by two and three cDNA clones, respectively. Interestingly, Tpm3 and Tpm4 genes encoding low-molecular-weight tropomyosins were not regulated by TGF- β (Table 1).

Treatment with a p38Mapk inhibitor suppressed induction of tropomyosins by 30–45% without a significant effect on calponin2 (Table 1). Northern blot analysis with rat TM3 cDNA, a product of the α -TM gene, revealed a 1.6-fold increase in the TM mRNA levels at 4 h reaching a 3.6-fold induction at 24 h of TGF- β 1 treatment (Figure 2a). Similar regulation was observed for α -actinin (Figure 2a). Cotreatment with a p38Mapk inhibitor reduced by 35% the induction of α -TM mRNA, without a significant effect on α -actinin1 (Figure 2a), suggesting that p38Mapk is involved in tropomyosin gene expression. Using RT-PCR we confirmed TGF- β -mediated regulation of calponin2 and that PAI-1, a known TGF- β -target gene, is regulated with kinetics similar to the newly identified TGF- β target genes (Figure 2b). The regulation of highly conserved α -TM and β -TM genes was further confirmed using RT-PCR with isoform specific primers (Figure 2c), because tropomyosin sequences are conserved. The specificity was also confirmed using cDNA clones for TM1, TM2, and

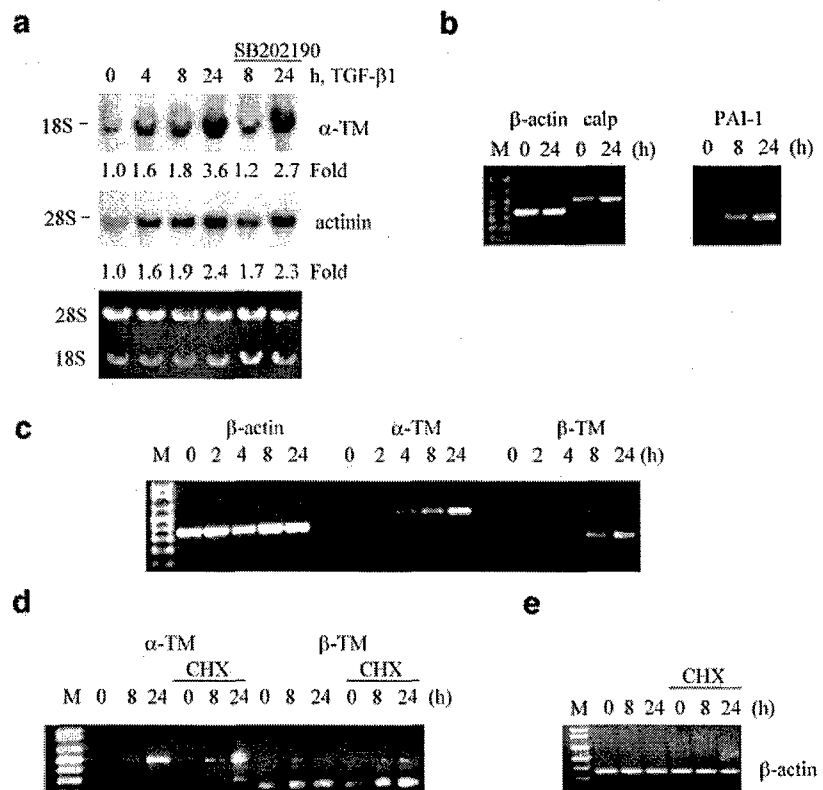


Figure 2. TGF- β regulates expression of genes encoding actin-binding proteins in NMuMG cells. (a) Northern blot analysis of TM2/3 and α -actinin1 mRNA levels in total RNA samples (15 μ g/lane) from NMuMG cells treated with 2 ng/ml TGF- β 1 in the absence or presence of 10 μ M SB202190. Blots were quantified using PhosphorImager. Bottom panel shows ethidium bromide staining of total RNA. (b–e) Analysis of β -actin, PAI-1, calponin2, and α - and β -tropomyosin transcripts by PCR with reverse transcription in total RNA samples from NMuMG cells treated with 2 ng/ml TGF- β 1. Where it is indicated, cells were treated with 10 μ g/ml cycloheximide (CHX) 1 h before addition of TGF- β 1.

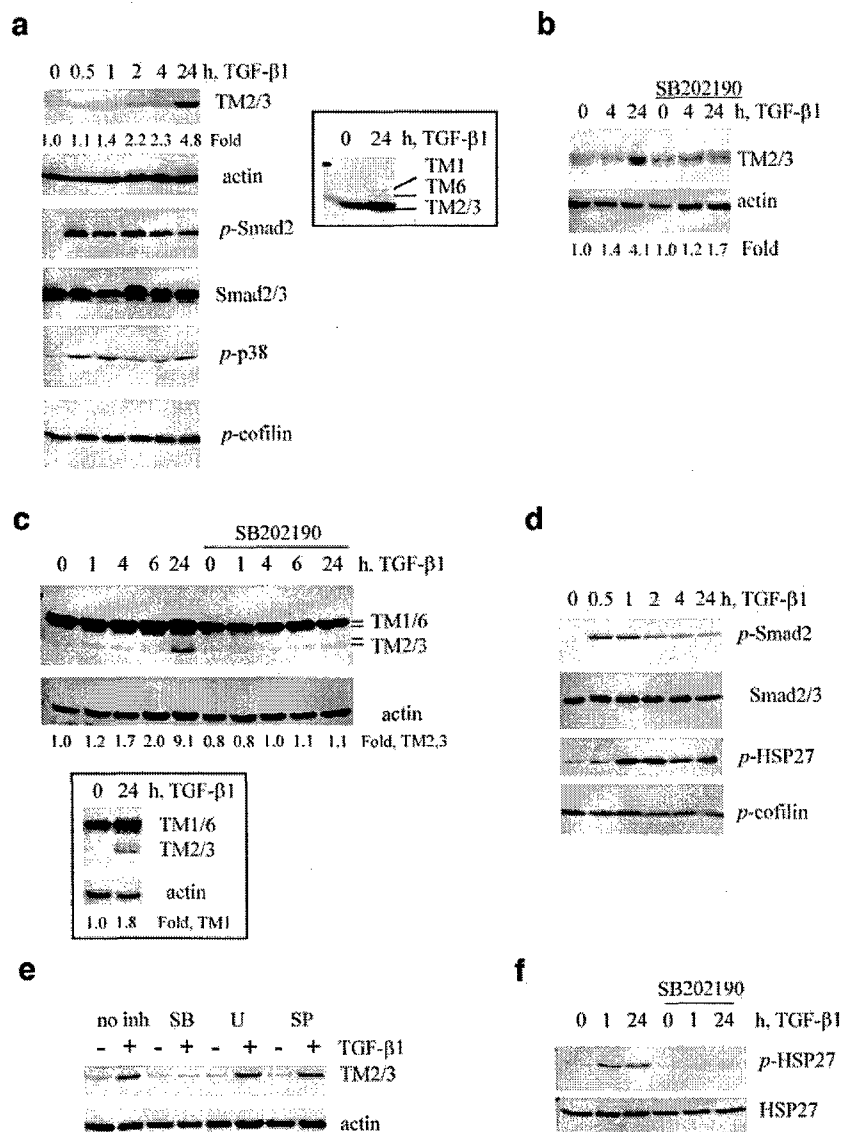


Figure 3. TGF- β regulates tropomyosins and HSP27 phosphorylation in epithelial cells. (a and b) Immunoblot analysis of actin and tropomyosins as well as phosphorylation of Smad2, HSP27, and cofilin using phospho-specific antibodies in NMuMG cells treated with 2 ng/ml TGF- β 1. Where it is indicated 10 μ M SB202190 was added 1 h before TGF- β treatment. Inset shows induction of TM1 and TM6 on a longer exposed film of the same immunoblot. (c–f) Immunoblot analysis of tropomyosin expression and phosphorylation of Smad2, p38Mapk, and cofilin in SiHa cells treated with 2 ng/ml TGF- β 1. Where it is indicated, the cells were cotreated 1 h before addition of TGF- β with 10 μ M SB202190 (SB), 5 μ M U0126 (U), or 15 μ M SP600125 (SP). Inset shows immunoblot with TM311 antibody using the same protein extracts as in c. Fold difference of tropomyosin levels relative to actin was estimated using NIH ImageJ software (<http://rsb.info.nih.gov/ni-image/>).

TM3 (our unpublished results). Examination of α - and β -TM mRNA levels in the cells cotreated with cycloheximide showed that TGF- β induction of these genes was not affected by cycloheximide, indicating that these genes are directly regulated by the TGF- β signaling pathway (Figure 2, d and e). Thus, we have identified tropomyosins α -actinin1 and calponin2 as novel TGF- β target genes that may account for TGF- β regulation of actin filament dynamics.

TGF- β Up-regulates Expression of Tropomyosins and Induces Phosphorylation of HSP27 in Epithelial Cells

In vertebrates, more than 10 different isoforms of high-molecular-weight tropomyosins are expressed from α - and β -TM genes and by alternative RNA splicing (Pittenger *et al.*, 1994). Tropomyosins form α -helical coil-coil dimers that bind along the length of the actin filaments interacting with 6–7 actin monomers are thought to be essential for the assembly and stabilization of actin filaments. (Ayscough, 1998). In this study we used the TM311 mAb recognizing tropomyosin 1 (TM1), a product of the β -TM gene, and

tropomyosin isoforms 2, 3, and 6 (TM2,3,6), products of the α -TM gene (Temm-Grove *et al.*, 1998). Immunoblot analysis with TM311 antibody followed by reblotting with anti- β -actin mAb showed that TGF- β induced a 4.8-fold increase in TM2 and TM3 in NMuMG cells (Figure 3a). Induction of TM1 and TM6 was also detected but required longer film exposures (Figure 3a, inset), suggesting that TM2 and TM3 are the main tropomyosin isoforms regulated by TGF- β at the protein level in NMuMG cells. The difference in the regulation of protein and mRNA levels of TM1 in response to TGF- β 1 is not obvious and may be related to a tight regulation of α/β tropomyosin isoforms (Robbins, 1998). Analysis of Smad2 and p38Mapk phosphorylation in the same cells showed activation of Smad and p38 signaling at 30 min and a sustained level for at least 24 h (Figure 3a). Inhibition of p38Mapk significantly reduced the induction of tropomyosins without affecting basal level expression (Figure 3b), suggesting a more profound effect of p38Mapk on tropomyosin protein than on mRNA (see Figure 2a). Inhibition of p38Mapk did not block phosphorylation of Smad2

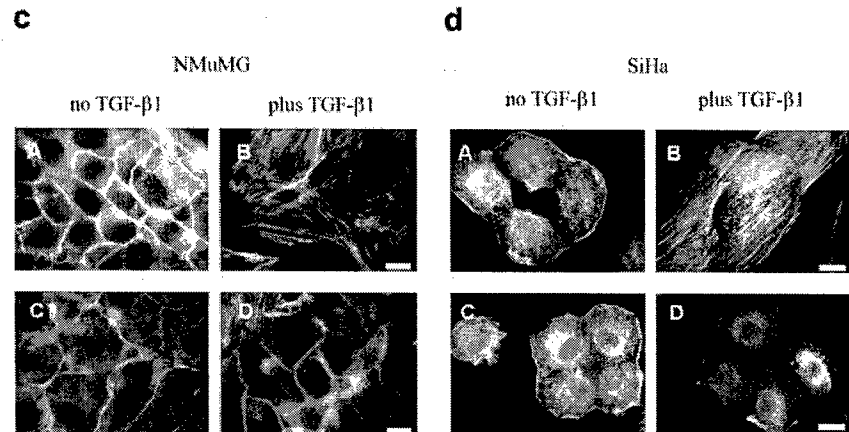
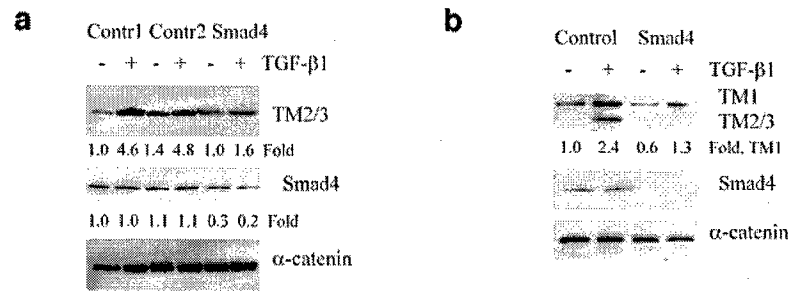


Figure 4. Smad signaling is required for TGF- β -induced expression of tropomyosins and stress fiber formation in epithelial cells. (a and b) Immunoblot analysis of tropomyosins and Smad4 in NMuMG cells (a) and SiHa cells (b) transfected with siRNAs against Smad4. (c and d) Actin filament staining with phalloidin-Alexa Green in NMuMG and SiHa cells transfected with control scramble siRNA (A and B) or siRNAs to Smad4 (C and D). The cells were treated with 2 ng/ml TGF- β 1 for 24 h. Scale bar, 10 μ m. Fold differences in tropomyosin and Smad4 levels relative to α -catenin were estimated using NIH ImageJ software.

and Smad-dependent transcription (Figure 1, b and c) and did not affect expression of calponin2 (Table 1).

TGF- β also mediated up-regulation of TMs and p38Mapk signaling in human cervical carcinoma SiHa cells (Figure 3, c-f), which respond to TGF- β with SFs (Bakin *et al.*, 2002). TGF- β stimulated a nearly twofold increase in TM1 and a ninefold increase in TM2/3 levels (Figure 3c). A comparable TGF- β -mediated induction of TMs and stress fiber formation were also observed in A549 lung epithelial cells (our unpublished results). SiHa cells express relatively low basal levels of TM2/3/6, but a high basal level of TM1 (Figure 3c). p38Mapk inhibitors blocked this up-regulation of TM2/3 (Figure 3, c and e), whereas inhibitors of MEK1/2 (U0126) and JNK, SP600125, did not (Figure 3e). Induction of the α -TM gene in SiHa cells at the mRNA level was confirmed by RT-PCR (see Figure 8a). We found that the activation of the Smad pathway and Smad3 levels were noticeably lower in SiHa cells (Figure 3d) than in NMuMG cells (Figure 3a). This may explain the moderate regulation of tropomyosins in SiHa cells compared with NMuMG cells and support the notion that Smads are involved in TGF- β -mediated regulation of tropomyosins.

The small heat shock protein HSP27 is a downstream target of p38Mapk signaling (Huot *et al.*, 1998). The HSP27 phosphorylation by p38Mapk-MAPKAP2/3 signaling at three serine residues increases a pool of HSP27 tetramers that facilitate actin polymerization (Huot *et al.*, 1998; Hedges *et al.*, 1999; Rogalla *et al.*, 1999). Using phospho-HSP27 antibodies we found that TGF- β stimulates a sustained phosphorylation of HSP27 in SiHa cells (Figure 3d). This is blocked by the p38Mapk inhibitor (Figure 3f). The actin filament dynamics is also controlled by the RhoA/ROCK/LIM-kinase pathway that regulates actin depolymerizing activity of ADF/cofilin by phosphorylation of a conserved serine3 in ADF/cofilin (Bamburg, 1999). Examination of cofilin phosphorylation in NMuMG (Figure 3a) and SiHa cells (Figure 3d) with phospho-Ser3-specific antibodies showed that levels of cofilin

phosphorylation did not change in response to TGF- β during stress fiber formation. These results indicate that TGF- β induction of stress fiber formation in epithelial cells is accompanied with an increase in expression of actin-binding proteins and p38Mapk-HSP27 signaling without a significant regulation of the ROCK/LIM-kinase/cofilin pathway.

The Smad Signaling Pathway Mediates Regulation of Tropomyosin Expression by TGF- β

To examine the involvement of Smads in TGF- β -induced expression of tropomyosins and SFs, we transfected NMuMG and SiHa cells with short interfering RNA duplexes (siRNA) against Smad4. Transfection of siRNAs significantly reduced Smad4 protein levels and TGF- β -induced expression of TM2/3 in NMuMG (Figure 4a). A more effective action of siRNAs was observed in SiHa cells where TM2/3 expression was prevented and TM1 level was reduced by 40–55% (Figure 4b). Staining of actin filaments with phalloidin-Alexa Green demonstrated a significant reduction in TGF- β -induced SFs in both cells lines transfected with siRNAs to Smad4 (Figure 4, c and d, panels C and D), compared with control siRNA (Figure 4, c and d, panels A and B). These results support a model that Smad signaling mediate induction of tropomyosin expression in response to TGF- β leading to formation of SFs in epithelial cells.

To test the contribution of specific Smads in the regulation of TMs and SFs, we used adenovirus-mediated expression of cDNAs encoding individual Smads in SiHa cells. These cells express low levels of Smad3 and Smad4 compared with NMuMG cells (Figure 3, a and d; Lee *et al.*, 2001). Flag-tagged Smad2 and Smad3 were expressed at comparable levels in SiHa cells infected with Smad-encoding adenoviruses (Figure 5, a and b). Smad3 significantly increased TGF- β -induced expression of tropomyosins compared with

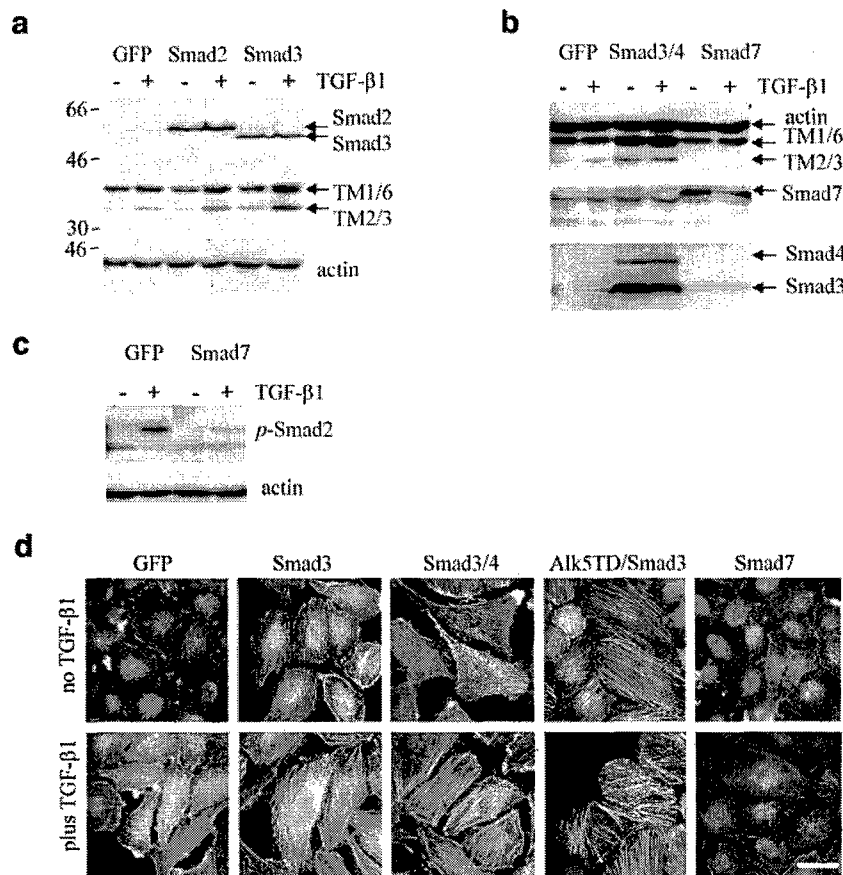


Figure 5. Smads mediate TGF- β -induced tropomyosin expression and stress fiber formation. (a and b) Tropomyosin expression in cells infected with adenoviruses encoding EGFP (GFP) and Flag-tagged Smad2, Smad3, Smad4, and Smad7. Cells were treated with 2 ng/ml TGF- β 1 for 24 h. (c) Inhibition of TGF- β 1-induced phosphorylation of Smad2 by adenoviral expression of Smad7 in SiHa cells. (d) Actin filaments staining in SiHa cells infected with adenoviruses: EGFP, Smad3, Smad4, Smad7, constitutively active Alk5T204D, or their combinations. The cells were treated with 2 ng/ml TGF- β 1 for 24 h. Scale bars, 15 μ m.

control EGFP-encoding adenovirus, whereas Smad2 exhibited only a moderate effect (Figure 5a). Coinfection with adenoviruses encoding Smad3 and Smad4 resulted in a marked increase of TMs even in the absence of added cytokine (Figure 5b), suggesting that Smad3 and Smad4 mediate TGF- β -regulated expression of tropomyosins. We next examined tropomyosin regulation by Smad7, an inhibitor of TGF- β signaling mediated by Smad2 and Smad3 (Massague, 1998). Expression of Smad7 inhibited TGF- β -induced expression of TM2/3 (Figure 5b) and phosphorylation of Smad2 (Figure 5c), whereas Smad6, an antagonist of bone morphogenic protein (BMP) signaling, had no effect (our unpublished results). In parallel, we examined actin filaments in SiHa cells infected with the adenoviral constructs (Figure 5d). As predicted, Smads that mediated enhancement of TM expression also increased SFs. Coexpression of Smad3 and Smad4 markedly increased SFs in the absence of exogenous TGF- β , which were further enhanced by the cytokine suggesting that other signaling events may also be involved in the assembly of stress fibers. Coexpression of constitutively active TGF- β type I receptor, Alk5T204D, and Smad3 resulted in SFs independent of exogenous TGF- β . Finally, expression of Smad7 significantly inhibited TGF- β -induced SF assembly (Figure 5e). These results demonstrate that Smad3 and Smad4 mediate TGF- β -induced expression of tropomyosins and SF formation.

Tropomyosins Are Required for SF Formation in Response to TGF- β

We confirmed that tropomyosins are localized with stable actin filaments resistant to Triton treatment. SiHa cells treated with TGF- β for 24 h were first incubated with Triton X-100 and then

fixed with 4% paraformaldehyde. Actin filaments were detected with phalloidin and tropomyosins with the TM311 antibody. Immunofluorescence microscopy showed that tropomyosins were localized along the actin microfilaments in a periodical pattern (Figure 6a).

To examine whether tropomyosins are required for TGF- β -mediated SF formation, SiHa cells were transfected with siRNA duplexes against tropomyosins (si-TMs) or a scrambled siRNA control. si-TMs effectively suppressed basal and TGF- β -induced expression of tropomyosins (Figure 6b). TGF- β induced SFs in cells transfected with a scrambled siRNA control (Figure 6c, panels A and B), whereas SFs were significantly reduced by si-TMs (Figure 6c, panels E and F). In control cells, TGF- β induced elongation of cells and localization of tropomyosins to actin filaments, whereas in the si-TM cells this response was significantly reduced (Figure 6c, panels C and D and G and H). A complementary experiment tested the gain-of-function by transfection of NMuMG and SiHa cells with expression vector for rat HA-tagged TM3. Expression of TM3 alone, without TGF- β treatment, was sufficient to induce SFs in both cell lines similar to cells treated with TGF- β (Figure 6d, red, panels C and D). Staining with fluorescein-labeled anti-HA antibody showed colocalization of HA-tagged TM3 with actin filaments (Figure 6d, panels A and B, and overlay). These results indicate that tropomyosins are both necessary and sufficient for TGF- β -induced stress fiber formation.

TGF- β Does Not Induce Stress Fibers but Stimulates Cell Migration in Metastatic Cells

Actin filaments are dynamic structures and stabilization of actin filaments limits cell movement. TGF- β induces stress fibers in NMuMG and SiHa cells and cells from both lines

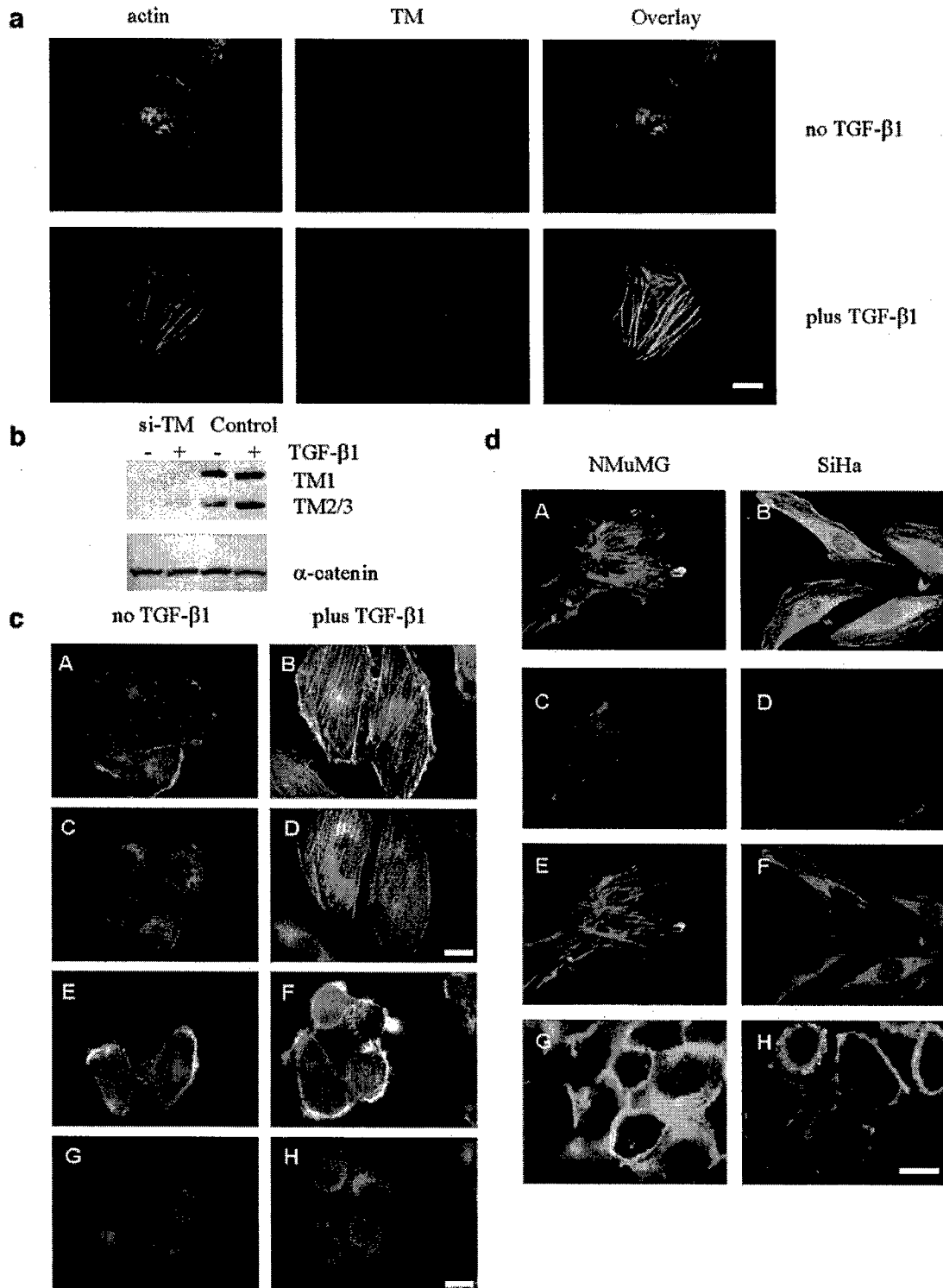


Figure 6. Tropomyosins are required for TGF- β -induced stress fiber formation. (a) Localization of tropomyosins (TM) to stable actin filaments (actin) resistant to 0.05% Triton X-100 treatment in SiHa cells untreated or treated with TGF- β 1 for 24 h. Scale bar, 10 μ M. (b) Suppression of tropomyosin expression in SiHa cells transfected with siRNA against TMs (si-TM) compared with a scrambled control. (c) Actin filaments (A, B, E, and F) and tropomyosin (C, D, G, and H) in SiHa cells, transfected with siRNA against tropomyosins (E-H) and a scrambled control siRNA (A-D). The cells were treated with 2 ng/ml TGF- β 1 for 24 h. Scale bar, 10 μ M. (d) Actin filaments in NMuMG and SiHa cells expressing HA-tagged TM3. Cells were stained with phalloidin-Texas Red and fluorescein-labeled anti-HA antibody (A-F). Overlay images are shown in panels E and F. Panels G and H show actin filaments and tropomyosins (TM311 antibody) in cells transfected with empty vector control. Scale bar, 15 μ m.

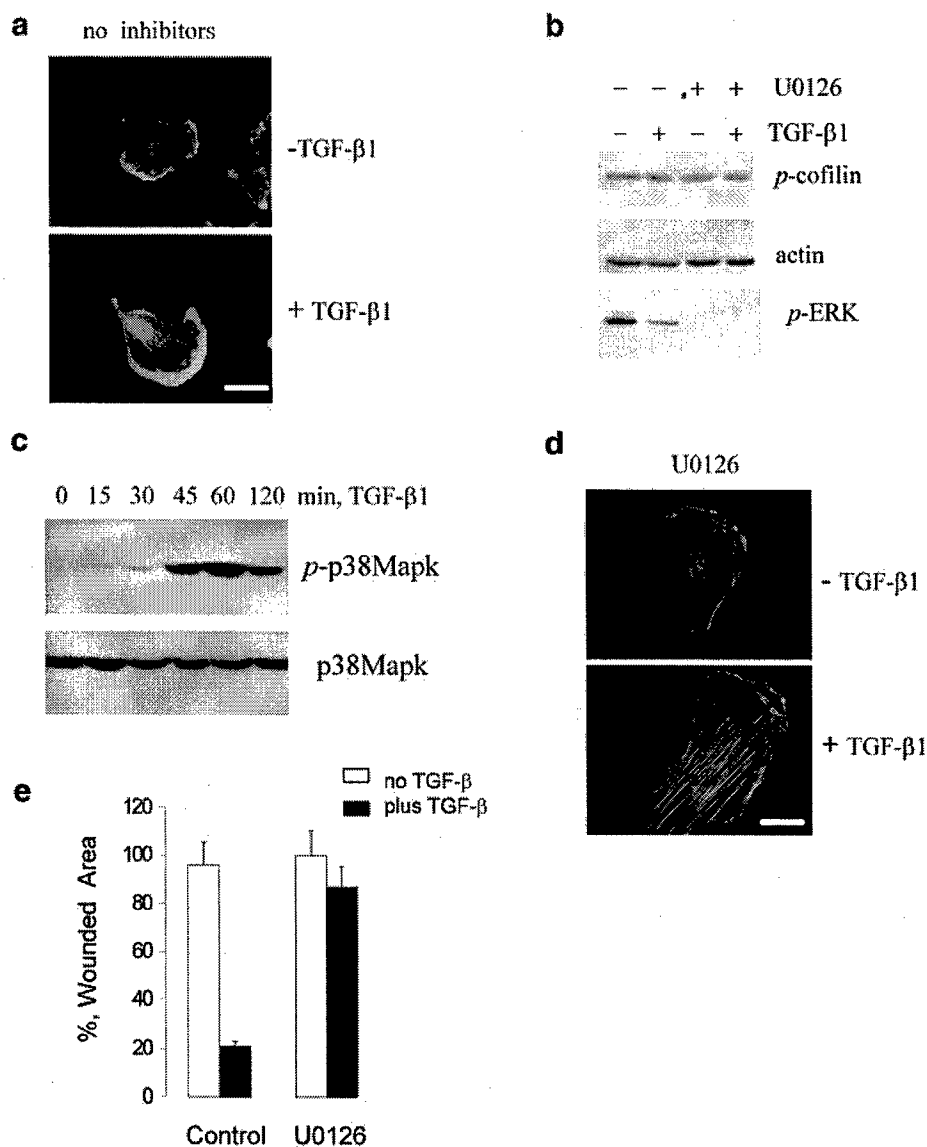


Figure 7. TGF- β and ERK signaling differentially regulate stress fiber formation. (a) Actin filaments staining in MDA-MB-231 cells treated with TGF- β 1 for 24 h. (b) Phosphorylation of cofilin and ERK1/2 in MDA-MB-231 cells cotreated with TGF- β 1 for 24 h and 5 μ M U0126. (c) Immunoblot analysis of p38Mapk phosphorylation in MDA-MB-231 cells treated with 2 ng/ml TGF- β 1. (d) Actin filaments staining in MDA-MB-231 cells cotreated with TGF- β 1 and 5 μ M U0126 for 24 h. (e) Wound closure in MDA-MB-231 cells treated with TGF- β 1 in the absence or presence of 5 μ M U0126. The experiment was done in triplicates and repeated at least two times. Scale bar, 20 μ M.

fail to migrate in response to TGF- β in a wound closure assay (our unpublished results). TGF- β has been shown to stimulate migration of metastatic breast cancer MDA-MB-231 cells in wound closure assay (Bakin *et al.*, 2002). We hypothesized that TGF- β -mediated stress fiber response is altered in MDA-MB-231 cells. Accordingly, treatment of MDA-MB-231 cells with TGF- β did not result in the formation of stress fibers (Figure 7a). MDA-MB-231 cells express TGF- β receptors, Smad factors, and respond to TGF- β 1 with activation of Smad, p38Mapk signaling, and regulation of gene expression (Bakin *et al.*, 2002; Dumont *et al.*, 2003 and Figure 7c). It has been reported that MDA-MB-231 cells have constitutively active Ras-ERK signaling (Kozma *et al.*, 1987; Ogata *et al.*, 2001), which may through the repression of the ROCK/LIM-kinase/cofilin pathway affect SF formation (Sa-

hai *et al.*, 2001; Pawlak and Helfman, 2002b). Thus, we examined phosphorylation of cofilin, a target of LIM kinase, in MDA-MB-231 cells. The immunoblot showed a relatively high basal level of the cofilin phosphorylation that was not modulated by TGF- β . Treatment of these cells with the MEK inhibitor did not affect the basal cofilin phosphorylation but blocked phosphorylation of ERK1/2 (Figure 7b). However, MEK inhibition significantly enhanced TGF- β -induced stress fiber formation (Figure 7d) and blocked TGF- β -mediated cell migration (Figure 7e). The TGF- β regulation of stress fibers was also restored by MEK inhibitor PD098059 and by inhibition of Raf kinase (our unpublished results). These results suggest that the ERK pathway suppresses TGF- β -mediated stress fiber formation in epithelial cells through a mechanism medi-

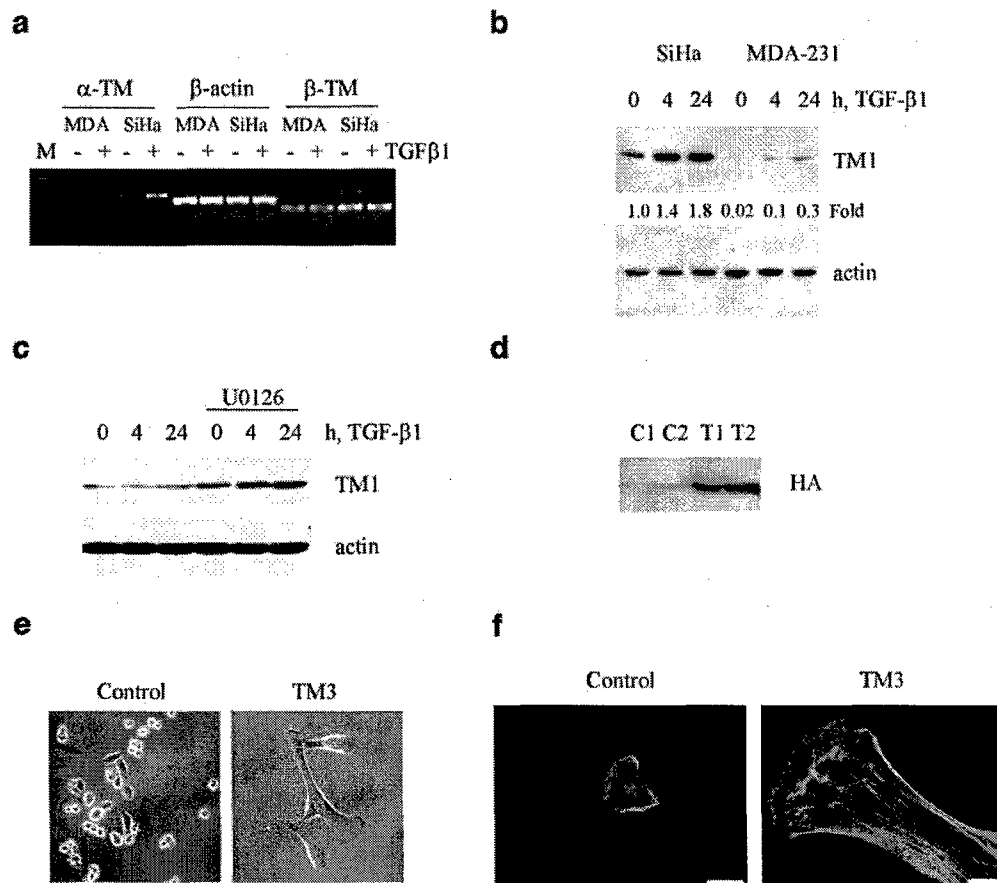


Figure 8. TGF- β and ERK signaling differentially regulates expression of tropomyosins. (a) Analysis of α -TM and β -TM transcripts by RT-PCR in total RNA samples from MDA-MB-231 (MDA) and SiHa cells treated with 2 ng/ml TGF- β 1 for 24h. (b) Immunoblot analysis of tropomyosin expression in protein extracts (35 μ g/lane) from SiHa and MDA-MB-231 cells treated with 2 ng/ml TGF- β 1. (c) Tropomyosin protein expression in MDA-MB-231 cells cotreated with TGF- β 1 and 5 μ M U0126 for 24h. (d) Detection of HA-tagged rat TM3 with anti-HA antiserum in protein extracts from two independent transfections of MDA-MB-231 cells with expression vector encoding HA-tagged rat TM3 (T1 and T2) or a control empty vector (C1 and C2). (e) Phase-contrast images show flattening and size increase in TM3-transfected MDA-MB-231 cells compared with control cells. (f) Immunofluorescence images show a marked increase in actin stress fibers in TM3-transfected MDA-MB-231 cells. Scale bar, 20 μ M. Fold differences in tropomyosin levels relative to actin were estimated using NIH ImageJ software.

ated by a pathway other than the ROCK/LIM-kinase/cofilin pathway.

Suppression of TGF- β -regulated Tropomyosin Expression by Ras-ERK Signaling in Metastatic MDA-MB-231 Cells

The Ras-ERK pathway has been implicated in suppression of tropomyosins and disruption of the actin cytoskeleton (Ljungdahl *et al.*, 1998; Shields *et al.*, 2002). We next examined whether the inability of TGF- β to induce stress fibers in MDA-MB-231 cells is associated with alteration of tropomyosin expression or function by Ras-ERK signaling. We compared expression of tropomyosins in MDA-MB-231 cells and SiHa cells, which show a low basal level of ERK phosphorylation. RT-PCR and immunoblot analysis showed that MDA-MB-231 cells express significantly less of TM1 mRNA and protein and undetectable levels of TM2/3 in comparison to SiHa cells (Figure 8, a and b). Treatment of MDA-MB-231 cells with the MEK inhibitor U0126 reduced ERK phosphorylation (Figure 7b), increased TGF- β -induced expression of TM1 (Figure 8c), and restored stress fibers (Figure 7d). Similar results were obtained with a Raf kinase inhibitor (our unpublished results). These data suggest that

Raf-ERK signaling down-regulates a basal and TGF- β -regulated expression of tropomyosin. Our findings also indicate that the α -tropomyosin gene is silenced in MDA-MB-231 cells.

We next examined whether ectopic expression of TM3, a product of the α -tropomyosin gene, will affect SFs and cell migration. MDA-MB-231 cells were transfected with expression vector encoding rat HA-tagged TM3 (Figure 8d) and analyzed for changes in cell morphology and actin filament assembly. Phase contrast images showed that TM3 expressing cells have a significant increase in cell size and a flatter more well-spread morphology compared with the refractile appearance of the parental cells or the control cells transfected with an empty vector (Figure 8e). Expression of TM3 markedly increased SFs in MDA-MB231 (Figure 8f) and inhibited cell motility assessed in the wound closure assay (our unpublished results). Interestingly, ectopic expression of either TM3 or TM2 inhibited proliferation of MDA-MB-231 cells increasing a number of multinucleated cells. We are currently developing inducible model to study effect of TMs on motility and growth of MDA-MB-231 cells. It has been also reported that overexpression of TM1 in MDA-MB-231 cells inhibits growth and motility of MDA-MB-231 cells (Raval *et al.*, 2003). Thus, overex-

pression of tropomyosins in metastatic MDA-MB-231 cells results in stress fibers and reduces cell motility. Collectively, the data presented above demonstrate that the Ras-ERK pathway inhibits TGF- β induction of stress fibers by suppressing expression of tropomyosins.

DISCUSSION

The molecular mechanism(s) underlying the prometastatic conversion of the TGF- β function is a major focus of current investigation by many research groups (reviewed in Derynck and Zhang, 2003; Roberts and Wakefield, 2003). In this study we found that the ability of TGF- β to induce stress fibers and, therefore, to control cell migration is significantly compromised in metastatic breast carcinoma cells. We provide evidence that tropomyosins are critical cellular components of Smad/p38Mapk-dependent actin stress fiber assembly in response to TGF- β in epithelial cells. We further show that the Ras-ERK pathway antagonizes TGF- β induction of tropomyosins and stress fibers. The restoration of tropomyosin expression results in stress fibers and reduces cell motility. These studies provide a direct causal link between TGF- β regulation of stress fibers and control of cell motility. These results suggest that the loss of the TGF- β stress fiber response in tumor cells is a critical step in prometastatic conversion of the TGF- β function.

We have investigated the mechanism of TGF- β regulation of actin filament dynamics and cell motility in normal and tumor epithelial cells. In untransformed epithelial cells TGF- β can rapidly induce membrane ruffling and actin polymerization at the cell edges, whereas a prolonged incubation with TGF- β results in the formation of stable actin filament bundles (stress fibers; Bakin *et al.*, 2002; Edlund *et al.*, 2002). We found that inhibition of either de novo protein synthesis or p38Mapk blocked TGF- β induction of stress fibers, suggesting that novel transcription/translation and p38Mapk signaling are required for TGF- β -mediated stress fiber formation. Consistent with these findings expression of kinase-inactive p38Mapk inhibited TGF- β induction of actin stress fibers (Bakin *et al.*, 2002). Here we show that concomitantly with stress fibers TGF- β induced a sustained activation of p38Mapk signaling and phosphorylation of HSP27 (Figure 3), a downstream target of the p38Mapk-MAPKAP kinase 2 pathway (Stokoe *et al.*, 1992). Phosphorylated HSP27 and the triple aspartate mutant mimicking HSP27 phosphorylation at Ser15, Ser78, and Ser82 form small oligomers and tetramers that facilitate de novo actin polymerization (Huot *et al.*, 1998; Hedges *et al.*, 1999; Rogalla *et al.*, 1999). We found that although inhibition of de novo protein synthesis blocked stress fibers, it did not affect p38Mapk signaling (Figure 1d), suggesting that p38Mapk-HSP27 signaling is required but not sufficient for TGF- β induction of stress fibers. This notion is also supported by previous studies that have suggested involvement of Smad signaling in stress fiber formation (Piek *et al.*, 1999a).

We identified several TGF- β target genes including α - and β -tropomyosins, α -actinin1, and calponin2 encoding actin-binding proteins implicated in the assembly of stress fibers. Among these genes, tropomyosins (TMs) have been shown to play a critical role in the stress fiber assembly by stabilizing actin filaments and preventing access of actin-severing factors gelsolin and ADF/cofilin to filamentous actin (Pawlak and Helfman, 2001). We found that TGF- β specifically up-regulates expression of α - and β -TM genes encoding high-molecular-weight TMs and does not regulate Tpm3 and Tpm4 genes encoding low-molecular-weight TMs (Table 1). The expression of TMs correlated with the ability of

TGF- β to induce stress fibers in several epithelial cell lines including NMuMG, SiHa, and A549 cells. Moreover, suppression of TM expression with siRNAs completely blocked TGF- β induction of stress fibers in mouse and human cell lines, whereas ectopic expression of TM2 and TM3, products of the α -tropomyosin gene, was sufficient to induce stress fibers even in the absence of the cytokine (Figures 6 and 7). This is the first demonstration that TMs play an essential role in TGF- β -induced stress fiber formation in mouse and human epithelial cell lines.

The TGF- β induction of TM expression depends on p38Mapk and Smad signaling. Inhibition of p38Mapk blocked expression of TM proteins without a significant effect on TM mRNA levels. These results suggest that p38Mapk is involved in posttranscriptional control of TM expression, although a recent study has implicated p38Mapk in regulation of TM mRNA in intestinal epithelial cells (Shields *et al.*, 2002). Silencing of Smad4 with siRNAs suppressed tropomyosin expression and blocked stress fiber formation (Figure 4), whereas adenoviral expression of Smad factors showed that Smad3 and Smad4 are required for the induction of tropomyosins and formation of stress fibers in epithelial cells (Figure 5). Importantly, inhibitory Smad7, but not Smad6, blocks TGF- β induction of TM expression and stress fiber formation. These results demonstrate that Smad3/Smad4 and p38Mapk are required for TGF- β -induced TM expression and stress fiber formation in epithelial cells.

Tropomyosins have been implicated in regulation of actin filament dynamics and control of cell motility (Pawlak and Helfman, 2001). Early studies have found that cell transformation by oncogenic Ras and Src leads to down-regulation of tropomyosins and disruption of actin stress fiber filaments (Leonardi *et al.*, 1982; Hendricks and Weintraub, 1984). Subsequently, it has been shown that ectopic expression of tropomyosins in Ras-transformed fibroblasts restores stress fibers and significantly reduces cell motility and cell growth (Takenaga and Masuda, 1994; Braverman *et al.*, 1996; Gimona *et al.*, 1996; Janssen and Mier, 1997). The importance of tropomyosins in the control of tumor invasion and metastasis is highlighted by several studies indicating that high-grade tumors of breast, prostate, bladder, and brain express significantly lower levels of tropomyosins than that of normal tissues (Franzen *et al.*, 1996; Wang *et al.*, 1996; Hughes *et al.*, 2003; Raval *et al.*, 2003). Thus, tropomyosins and thereby stress fibers may play a critical role in the TGF- β control of tumor invasion and metastasis. In support of this idea, we found that TGF- β induction of tropomyosins and stress fibers is markedly reduced in metastatic breast cancer MDA-MB-231 cell line (Figure 7). MDA-MB-231 cells express constitutively active Ras-ERK signaling (Kozma *et al.*, 1987; Ogata *et al.*, 2001) that has been implicated in down-regulation of tropomyosins and disruption of actin stress fibers (Ljungdahl *et al.*, 1998; Shields *et al.*, 2002). We found that pharmacological inhibition of the Raf-ERK pathway significantly increased basal and TGF- β -induced levels of TM1, restored TGF- β induction of stress fibers, and inhibited cell motility without any effect on phosphorylation of cofilin (Figures 7 and 8). These results can be attributed at least in part to changes in Smad signaling. In fact, recent studies have shown that the Ras-ERK pathway attenuates Smad signaling by affecting the subcellular localization of Smad2 and Smad3 (Kretzschmar *et al.*, 1999) and by inducing a proteasome-mediated degradation of Smad4 (Saha *et al.*, 2001). We also found that the α -TM gene is not expressed in MDA-MB-231 cells. Our unpublished data indicate that the CpG island in the proximal promoter of the human α -TM

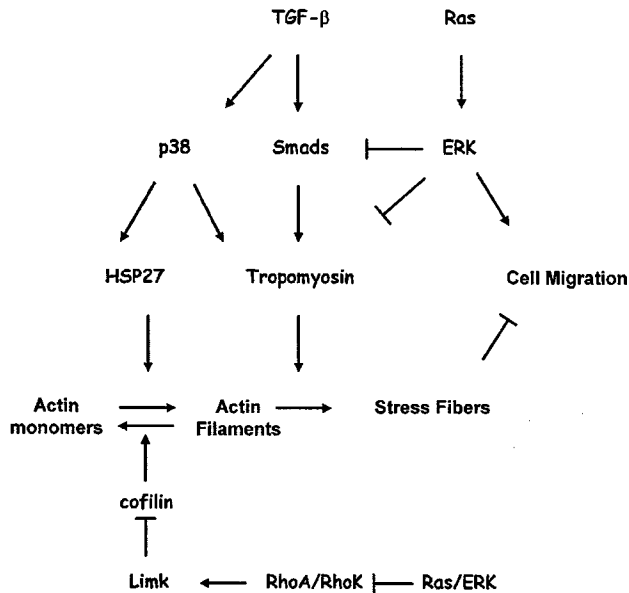


Figure 9. Opposing roles of the TGF- β and Ras-ERK signaling pathways in the regulation of actin filament dynamics and cell motility.

gene is methylated. The significance of this finding is currently under investigation. Importantly, ectopic expression of TM3 in MDA-MB-231 cells resulted in stress fibers (Figure 8) and severely affected cell motility. This finding is consistent with studies in rat NRK 1569 cells and mouse NIH-3T3 cells (Gimona *et al.*, 1996; Janssen and Mier, 1997). Our previous results suggest that Ras-ERK signaling does not affect TGF- β induction of membrane ruffling at the leading edge (Bakin *et al.*, 2002). Thus, Ras-ERK signaling suppresses TGF- β induction of tropomyosin expression and stress fibers leading to more motile and invasive phenotype.

The Rho-like GTPases, RhoA, Rac1, and CDC42, have been implicated in TGF- β mediated stress fiber formation (Moustakas and Stournaras, 1999; Bhowmick *et al.*, 2001; Bakin *et al.*, 2002; Edlund *et al.*, 2002). These GTPases through RhoA-ROCK/Rho-kinase and Rac/CDC42-Pak signaling can activate LIM kinases that negatively regulate ADF/cofilins by phosphorylating a conserved Serine3 in ADF/cofilins (Gungabissoon and Bamburg, 2003). ADF/cofilins regulate the turnover rates of actin filaments by promoting the dissociation of actin filaments into monomers (Bamburg, 1999). Thus, Rho-like GTPases through LIM kinases may contribute to stress fiber formation by inhibiting actin depolymerization. In this study we found that phosphorylation of cofilin was not modulated by TGF- β in three different cell lines, suggesting that the ROCK/LIM-kinase/cofilin pathway is not a target in TGF- β induction of stress fibers. Tropomyosins bound to filamentous actin prevent access of ADF/cofilins to actin filaments, thereby stabilizing actin filaments and reducing actin dynamics (Ono and Ono, 2002). In addition to tropomyosins, calponin2 and α -actinin1, two other TGF- β targets identified in this study, have been also implicated in stabilization of actin filaments (Panasenko and Gusev, 2001; Gimona *et al.*, 2003). It remains to be determined whether, in addition to tropomyosins, calponins and α -actinin play a role in formation of stress fibers in response to TGF- β by blocking ADF/cofilins and gelsolin from binding to actin filaments.

Our studies demonstrate an important role of tropomyosins in TGF- β regulation of stress fibers and cell migration (Figure 9). ERK signaling may inhibit TGF- β induction of stress fibers by suppressing Smad-dependent expression of tropomyosins. In addition, ERK signaling may affect stress fibers by disabling the RhoA/ROCK pathway (Pawlak and Helfman, 2002a, 2002b; Sahai *et al.*, 2001; Vial *et al.*, 2003). The suppression of tropomyosin expression by oncogenic Src (Hendricks and Weintraub, 1984) may also contribute to the cooperation of TGF- β and Src in tumorigenesis (Sieweke *et al.*, 1990). Thus, our study support an idea that the acquisition of metastatic phenotype by tumor cells results from the action of oncogenes and tumor suppressor genes regulating cell proliferation and survival (Bernards and Weinberg, 2002). Our results suggest that loss of TGF- β induction of stress fibers is an essential characteristic of a prometastatic conversion of TGF- β function and restoration of this response represents a potential target for the development of effective antimetastatic therapies.

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ORIGINAL PAPER

Silencing of the Tropomyosin-1 gene by DNA methylation alters tumor suppressor function of TGF- β

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Loss of actin stress fibers has been associated with cell transformation and metastasis. TGF- β induction of stress fibers in epithelial cells requires high molecular weight tropomyosins encoded by TPM1 and TPM2 genes. Here, we investigated the mechanism underlying the failure of TGF- β to induce stress fibers and inhibit cell migration in metastatic cells. RT-PCR analysis in carcinoma cell lines revealed a significant reduction in TPM1 transcripts in metastatic MDA-MB-231, MDA-MB-435 and SW620 cell lines. Treatment of these cells with demethylating agent 5-aza-2'-deoxycytidine (5-aza-dC) increased mRNA levels of TPM1 with no effect on TPM2. Importantly, 5-aza-dC treatment of MDA-MB-231 cells restored TGF- β induction of TPM1 and formation of stress fibers. Forced expression of TPM1 by using Tet-Off system increased stress fibers in MDA-MB-231 cells and reduced cell migration. A potential CpG island spanning the TPM1 proximal promoter, exon 1, and the beginning of intron 1 was identified. Bisulfite sequencing showed significant cytosine methylation in metastatic cell lines that correlated with a reduced expression of TPM1. Together these results suggest that epigenetic suppression of TPM1 may alter TGF- β tumor suppressor function and contribute to metastatic properties of tumor cells.

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Keywords: metastasis; TGF- β ; tropomyosin; Smad; DNA methylation

Introduction

The transforming growth factor beta (TGF- β) signaling pathway is a major cellular growth inhibitory and proapoptotic pathway in epithelial, endothelial, hematopoietic and other cell types (Roberts and Wakefield, 2003). Clinical and experimental studies indicate that TGF- β can enhance the metastatic behavior of the tumor cells (Saito *et al.*, 2000; Derynck *et al.*, 2001). This apparent paradox has been associated with a progressive decline in TGF- β antitumorigenic function

and a gain of protumorigenic activities including induction of epithelial to mesenchymal transition (EMT), cell migration and invasion during tumor progression (Derynck *et al.*, 2001; Roberts and Wakefield, 2003). Oncogenic Ras, Src and ErbB2 as well as alterations in TGF- β signaling mediated by Smads, mitogen-activated protein (MAP) kinases, Rho kinases and Akt/PKB are thought to contribute to the metastatic phenotype (Derynck *et al.*, 2001; Roberts and Wakefield, 2003).

The actin cytoskeleton plays a central role in the regulation of cellular processes linked to metastasis including cell proliferation, apoptosis, anchorage-independent cell growth, cell migration, and invasion (Jaffe and Hall, 2002). Oncogenic transformation alters regulation and organization of the actin cytoskeleton by suppressing actin-binding proteins involved in stabilization of actin microfilaments (Pawlak and Helfman, 2001) or by disabling the RhoA/ROCK pathway (Sahai *et al.*, 2001; Pawlak and Helfman, 2002a, b; Vial *et al.*, 2003). In normal and tumor epithelial cells, TGF- β regulates dynamics of the actin cytoskeleton (Bakin *et al.*, 2002; Edlund *et al.*, 2002) through mechanisms involving Smads (Piek *et al.*, 1999), Rho kinase (Bhowmick *et al.*, 2001) and p38 MAP kinase (Hannigan *et al.*, 1998; Bakin *et al.*, 2002; Edlund *et al.*, 2002). A recent study has shown that high molecular weight (HMW) tropomyosins are critical components of Smad/p38MAPK-dependent actin stress fiber formation in response to TGF- β in epithelial cells (Bakin *et al.*, 2004). In normal and tumor nonmetastatic epithelial cells TGF- β upregulates HMW tropomyosins through p38MAPK- and Smad-dependent mechanisms leading to the formation of stable actin filaments (stress fibers) and reduction of cell motility (Bakin *et al.*, 2004). Tropomyosins have been implicated in the assembly and stabilization of actin filaments and control of cell motility (Pawlak and Helfman, 2001). Tropomyosins form α -helical coil-coil dimers that bind along the length of the actin filaments interacting with 6–7 actin monomers and stabilize actin filaments (Ayscough, 1998). In vertebrates, more than 10 different isoforms of HMW tropomyosins are expressed from TPM1 (α -TM) and TPM2 (β -TM) genes, and by alternative RNA splicing (Pittenger *et al.*, 1994). Early studies have shown that cell transformation by oncogenic Ras and Src leads to downregulation of HMW tropomyosins and

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disruption of actin stress fiber filaments (Leonardi *et al.*, 1982; Hendricks and Weintraub, 1984). Subsequently, it has been found that ectopic expression of HMW tropomyosins in Ras-transformed fibroblasts restores stress fibers and significantly reduces cell motility and cell growth (Braverman *et al.*, 1996; Gimona *et al.*, 1996; Janssen and Mier, 1997). Several studies have reported that high-grade tumors of breast, prostate, bladder and brain express significantly lower levels of HMW tropomyosins compared to normal tissues (Franzen *et al.*, 1996; Wang *et al.*, 1996; Hughes *et al.*, 2003; Raval *et al.*, 2003; Pawlak *et al.*, 2004). Thus, HMW tropomyosins and thereby stress fibers may play a critical role in control of tumor invasion and metastasis.

Previously, we have reported that TPM1 was not regulated by TGF- β 1 in metastatic breast cancer MDA-MB-231 cells (Bakin *et al.*, 2004), although these cells express TGF- β receptors and Smad signaling components (Dumont *et al.*, 2003). Here we investigated the mechanism underlying suppression of TPM1 gene expression in metastatic cells. Our data show that suppression of TPM1 gene expression is associated with hypermethylation of CpG sites within the TPM1 promoter in metastatic breast and colon cell lines. Demethylation of MDA-MB-231 cells restored TGF- β induction of TPM1 expression and stress fiber formation. Expression of tropomyosin in MDA-MB-231 cells using Tet-Off system inhibited cell migration. Thus, epigenetic inactivation of TPM1 may alter TGF- β tumor suppressor function and contribute to acquisition of malignant phenotype.

Results

Stress fiber formation in response to TGF- β in epithelial cells

We investigated TGF- β induction of tropomyosins and actin stress fibers in human lung epithelial A549 cells, cervical carcinoma SiHa cells and breast carcinoma MDA-MB-231 cells (Figure 1a). Treatment of cells with TGF- β resulted in the formation of stress fibers in SiHa and A549 cells, but not in MDA-MB-231 cells (Figure 1a). Tropomyosin expression was analysed with the TM311 monoclonal antibody recognizing the N-terminal epitope in tropomyosin 1 (TM1), a TPM2 gene product, and tropomyosin isoforms 2, 3 and 6 (TM2,3,6), products of the TPM1 gene (Temm-Grove *et al.*, 1998). Immunoblotting showed that TGF- β stimulated expression of TM1-3, and 6 in A549 and SiHa cells. TPM1 gene products were not expressed in MDA-MB-231 cells, while TM1, a TPM2 product, was expressed at significantly lower levels compared to SiHa cells (Figure 1b). RT-PCR with gene-specific primers showed TPM2 mRNA expression in both cell lines, whereas TPM1 mRNA was induced by TGF- β in SiHa and A549 cells but not in MDA-MB-231 cells (Figure 1c). MDA-MB-231 cells express TGF- β receptors, Smad factors, and respond to TGF- β with activation of Smad and p38 MAPK signaling, as well

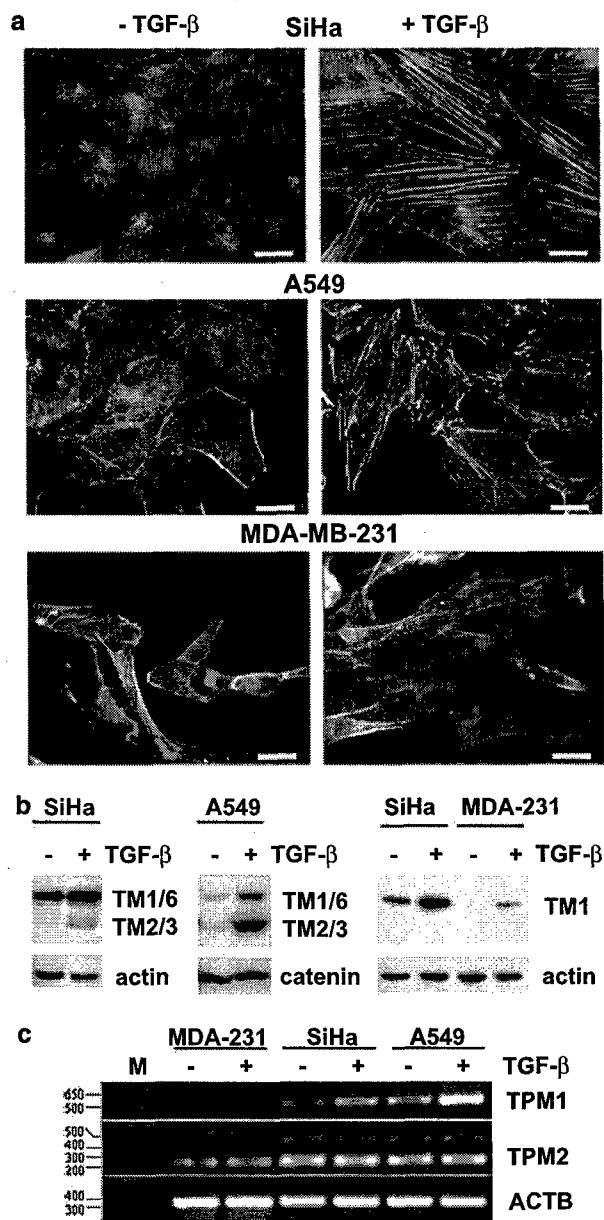


Figure 1 Actin stress fibers and tropomyosin expression are upregulated by TGF- β 1 in epithelial cells. (a) Staining of actin filaments with phalloidin Alexa Green. Induction of stress fibers after 24 h incubation with TGF- β 1. Scale bar, 20 μ m. (b) Increased accumulation of tropomyosins in SiHa, A549, and MDA-MB-231 cells after TGF- β 1 treatment for 24 h, as shown by immunoblotting with the TM311 antibody. (c) RT-PCR analysis of mRNA levels for TPM1, TPM2 and β -actin in MDA-MB-231, SiHa and A549 cell lines treated with TGF- β 1 for 24 h

as regulation of gene expression (Bakin *et al.*, 2002; Dumont *et al.*, 2003). Hence, the absence of TPM1 expression in MDA-MB-231 cells could arise from genetic or epigenetic abnormalities in the TPM1 gene or because tissue-specific differences in cell lines used.

Together, these results indicate that the ability of TGF- β to induce stress fibers in epithelial cells correlates

with expression of HMW tropomyosins, and that the absence of TGF- β -induced stress fibers in MDA-MB-231 cells may be associated with loss of TPM1 expression and low expression of TPM2.

Suppression of TPM1 expression in metastatic breast cancer cell lines by DNA methylation

To rule out the involvement of tissue-specific differences, mRNA levels of TPM1 and TPM2 were compared in several commonly used human mammary and colon epithelial cell lines by RT-PCR. TPM1 transcripts were expressed in normal MCF10A and several breast carcinoma cell lines, but were absent or found at lower levels in metastatic MDA-MB-231 and MDA-MB-435 cell lines (Figure 2a). TPM2 transcripts were present at comparable levels in all examined breast cell lines (Figure 2a). Of note, although TPM2 was expressed in MCF7 cells no TM1 protein was detected by immunoblotting (A Bakin, unpublished; Bharadwaj and Prasad, 2002), suggesting a post-transcriptional regulation of the TPM2 gene expression in these cells. To further test a correlation between TPM1 expression and a metastatic behavior of tumor cells, tropomyosin transcripts were examined in two colon cancer cell lines established from primary tumor (SW480) and a metastatic lesion (SW620) of the same patient (Gagos et al., 1995). TPM2 was expressed in both cell lines, whereas TPM1 transcripts were below a detection level in metastatic SW620 cells (Figure 2d). These results indicate that TPM1 is expressed in normal mammary epithelial cells, but significantly reduced or absent in metastatic cell lines.

To determine whether DNA methylation contributes to suppression of the TPM1 gene in breast cancer MDA-MB-231 and MDA-MB-435 cell lines, tumor cells were treated with demethylating agent 5-aza-dC. RT-PCR analysis showed that 5-aza-dC treatment restored TPM1 expression in MDA-MB-435 cells to the levels comparable with MCF10A and MCF7 cell lines (Figure 2b). Treatment with 5-aza-dC of MDA-MB-231 cells increased TPM1 mRNA levels that were further upregulated by TGF- β 1 (Figure 2c). TPM2 transcripts were reduced by approximately 40% in 5-aza-dC-treated cells (Figure 2d), although, TPM2 protein level (TM1 isoform) was induced (Figure 3b), suggesting indirect effect of 5-aza-dC on TPM2 expression. These results indicate that DNA methylation may be involved in suppression of the TPM1 gene expression in metastatic carcinoma cell lines, and that low expression of HMW tropomyosins correlates with metastatic phenotype of tumor cells.

Re-expression of TPM1 in MDA-MB-231 cells results in stress fiber formation

To verify RT-PCR data and to test effects of TPM1 expression on stress fiber formation, we examined tropomyosin proteins and actin filaments in 5-aza-dC-treated MDA-MB-231 cells. Smad2/3 phosphorylation in response to TGF- β 1 was not affected in MDA-MB-

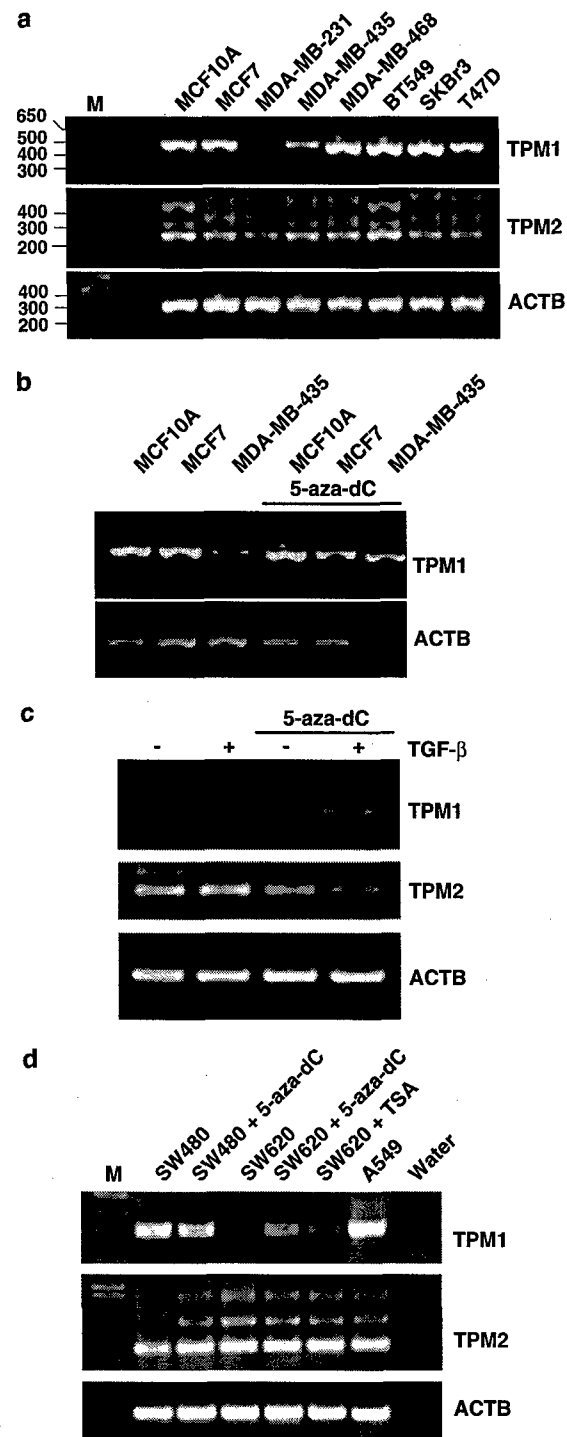


Figure 2 RT-PCR analysis of TPM1 and TPM2 expression. (a) TPM1 and TPM2 mRNA levels in human mammary epithelial cell lines. In (a-d), β -actin (ACTB) was used as a control. (b) Effect of 5-aza-dC treatment on TPM1 mRNA levels in human mammary epithelial cell lines. (c) TPM1 and TPM2 expression in MDA-MB-231 cells treated with 5-aza-dC and TGF- β 1. (d) TPM1 and TPM2 mRNA in human colon carcinoma SW480 and SW620 cells, and lung carcinoma A549 cells

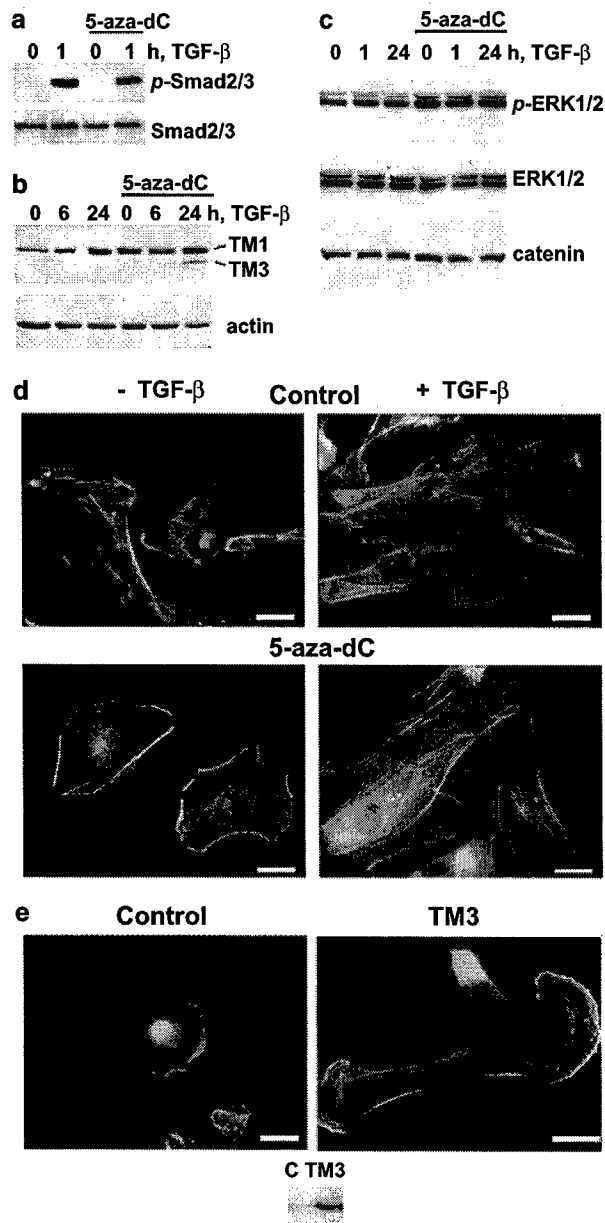


Figure 3 Restoration of TGF- β -mediated induction of tropomyosins and stress fibers in MDA-MB-231 cells treated with 5-aza-dC. (a) Phosphorylation of Smad2/3 in cells treated with 5-aza-dC and TGF- β 1. (b) Immunoblot detection of TM proteins using TM311 antibody in cells treated with TGF- β 1. (c) Immunoblot with antibodies to phospho-ERK1/2, ERK1/2, and α -catenin in cells treated with 2 ng/ml TGF- β 1. (d) Phalloidin staining of actin microfilaments in control and 5-aza-dC-treated MDA-MB-231 cells incubated \pm 2 ng/ml TGF- β 1 for 24 h. (e) Actin microfilaments in MDA-MB-231 cells transfected with plasmid encoding HA-tagged-TM3 and empty vector control. Inset shows immunoblot with anti-HA antibody. Scale bar, 20 μ m.

231 cells by 5-aza-dC treatment (Figure 3a). Immunoblot analysis revealed induction of TM2/3 isoforms by TGF- β 1 in 5-aza-dC-treated MDA-MB-231 cells and enhancement of basal TM1 expression (Figure 3b). Since MEK-ERK signaling may affect tropomyosin

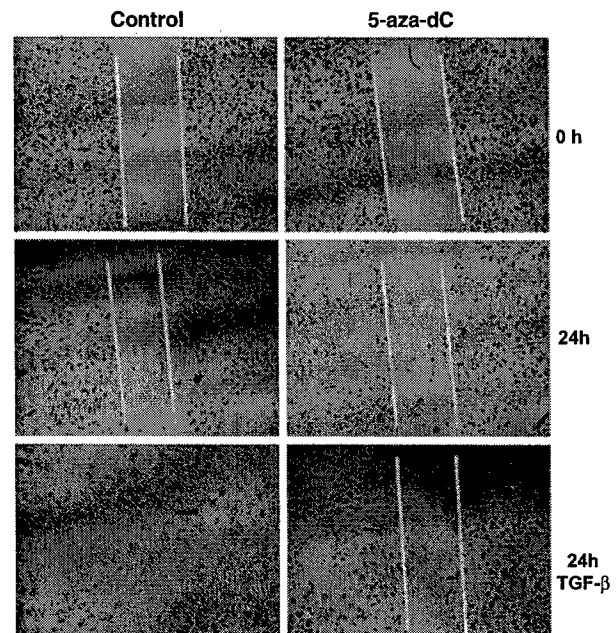


Figure 4 Inhibition of cell migration by 5-aza-dC treatment. Wound closure in monolayers of control and 5-aza-dC-treated MDA-MB-231 cells in the presence or absence of 1 ng/ml TGF- β 1. Microphotographs were taken at \times 100 magnification. Lines show wound edges. The wound is closed in control cells incubated with TGF- β 1 but not in 5-aza-dC-treated cells.

expression (Bakin *et al.*, 2004), we examined phosphorylation and total levels of ERK1/2 in control and 5-aza-dC-treated MDA-MB-231 cells. Immunoblots showed that 5-aza-dC-treatment did not decrease levels of phospho-ERK1/2 and total ERK1/2 (Figure 3c). This result indicates that induction of TPM1 in 5-aza-dC-treated MDA-MB-231 cells cannot be explained by inhibition of ERK1/2.

Phalloidin staining showed higher levels of microfilaments in 5-aza-dC-treated MDA-MB-231 cells compared to control cells that were further enhanced by treatment with TGF- β 1 (Figure 3d). Importantly, 5-aza-dC treatment inhibited wound closure in MDA-MB-231 cell monolayers in response to TGF- β 1, indicating that de-methylation reduces cell migration (Figure 4).

We then asked whether ectopic expression of TM3 encoded by the TPM1 gene, in MDA-MB-231 cells is sufficient for stress fiber formation. Rat TM3 cDNA (Gimona *et al.*, 1996) was transiently transfected in MDA-MB-231 cells and expression of HA-tagged TM3 was confirmed by immunoblotting (Figure 3e, inset). Actin microfilament fibers were significantly increased in TM3-expressing cells even in the absence of TGF- β 1 compared to control cells (Figure 3e). Selection of stable TM3 clones failed as TM3-expressing cells did not grow and eventually died due to block in cytokinesis and accumulation of multiple nuclei. To overcome this problem we generated MDA-MB-231 Tet-Off cells with inducible expression of rat TM3. Expression of tropomyosin was induced by incubating the Tet-Off cells in the absence of doxycycline (Figure 5a). The achieved

level of tropomyosin was nearly 50% of MCF7 cells. TM3-expressing cells showed an increase in actin microfilament fibers and reduction in actin ruffles at the cell edges compared to cells grown in the presence of doxycycline when TM3 is not expressed (Figure 5b). Migration of TM3-expressing cells in transwell assay was reduced approximately 1.7-fold (Figure 5c, d).

These results suggest that DNA methylation is involved in silencing of the TPM1 gene in MDA-MB-

231 cells, and that re-expression of TPM1 leads to stress fibers and reduces cell migration.

Identification of a CpG island in the TPM1 promoter

The TPM1 gene is expressed in most tissues and its disruption in mice results in early embryonic lethality, suggesting that it is critically involved in development (Robbins, 1998). We hypothesized that TPM1 may contain a CpG island in the promoter. As no studies have reported on the DNA structure of human TPM1 gene, database-mining was performed to define the exon-intron structure, promoter and CpG island of human TPM1 gene. Comparison of the 5' cDNA sequences of mammalian TPM1 with mouse and human genomic DNA (gDNA) sequences obtained from BLAT (<http://genome.ucsc.edu/cgi-bin/hgBlat>) using ClustalW (<http://www.ebi.ac.uk/clustalw>) revealed that the 5'-end of the mouse cDNA is located within 21 bp from a TATATA sequence, which is identical in both human and mouse gDNA sequences (Figure 6a). The potential TATA box is located in the highly conserved gDNA region containing several Sp1-like binding sequences.

The analysis of gDNA sequences using BLAT revealed two large CpG islands in both human *TPM1* and mouse *Tpm1*, with one near exon 1 and the other in a region further downstream, in a sequence aligning with rat exon 1b (GenBank accession M34137). The CpG islands of both human and mouse TPM1 conformed to the CpG island definition (Antequera and Bird, 1993; Cross and Bird, 1995). The two human TPM1 CpG islands are of length 1.829 kb with 163 CpG sites, a G/C content of 67% and a CpG:GpC ratio of 0.8; and 1.208 kb with 110 CpG sites, a G/C content of 70% and a CpG:GpC ratio of 0.71, covering putative exons 1 and 1b, respectively. The human 1.8 kb TPM1 CpG island encompasses the transcription start, exon 1 and a portion of intron 1 (Figure 6a). This region contains several potential Sp1-binding sites overlapping with CpG dinucleotides. To test whether Sp1 is involved in TPM1 expression, mouse mammary epithelial NMuMG cells were treated with TGF- β 1 in the presence of MTA, which inhibits Sp1 binding to DNA and TGF- β 1-induced gene expression (Chung et al., 1996; Albo et al., 1997; Greenwel et al., 1997; Park et al., 2000). Treatment with 200 nM MTA significantly reduced basal and TGF- β 1-induced levels of TPM1 transcripts, suggesting involvement of Sp1-like factors in TGF- β 1-mediated induction of the TPM1 expression (Figure 6b).

Thus, both human and mouse TPM1 genes contain CpG islands, and the proximal promoter CpG island includes a putative transcription start site, the TATA box and Sp1-like sites.

Methylation of the TPM1 CpG island in metastatic cell lines

We investigated DNA methylation of the human 1.8 kb TPM1 CpG island by the bisulfite-sequencing method (Frommer et al., 1992). PCR primers were designed to

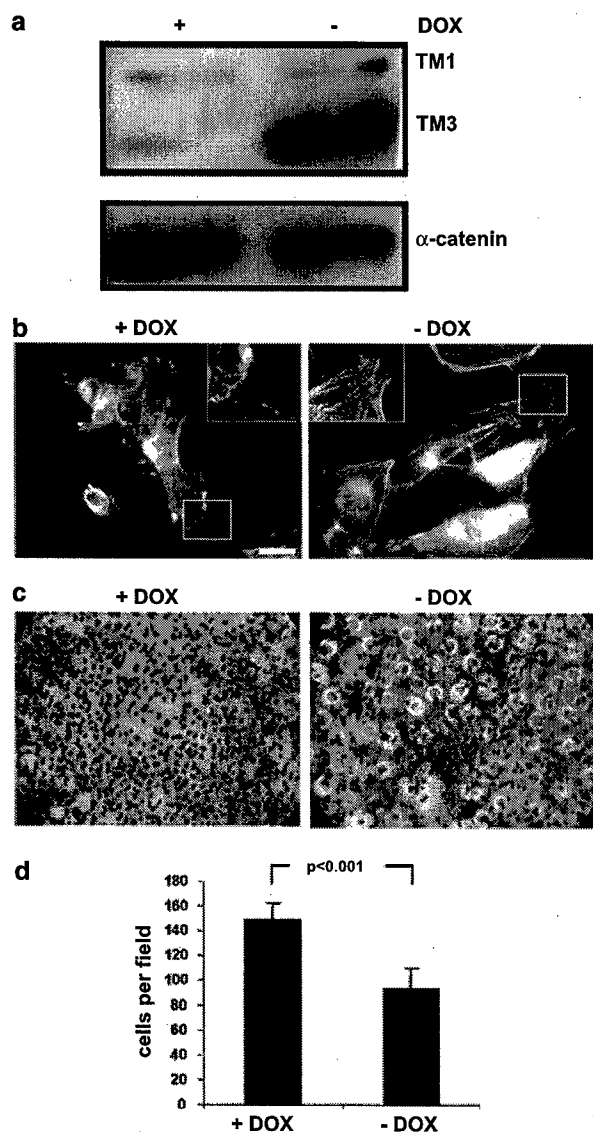
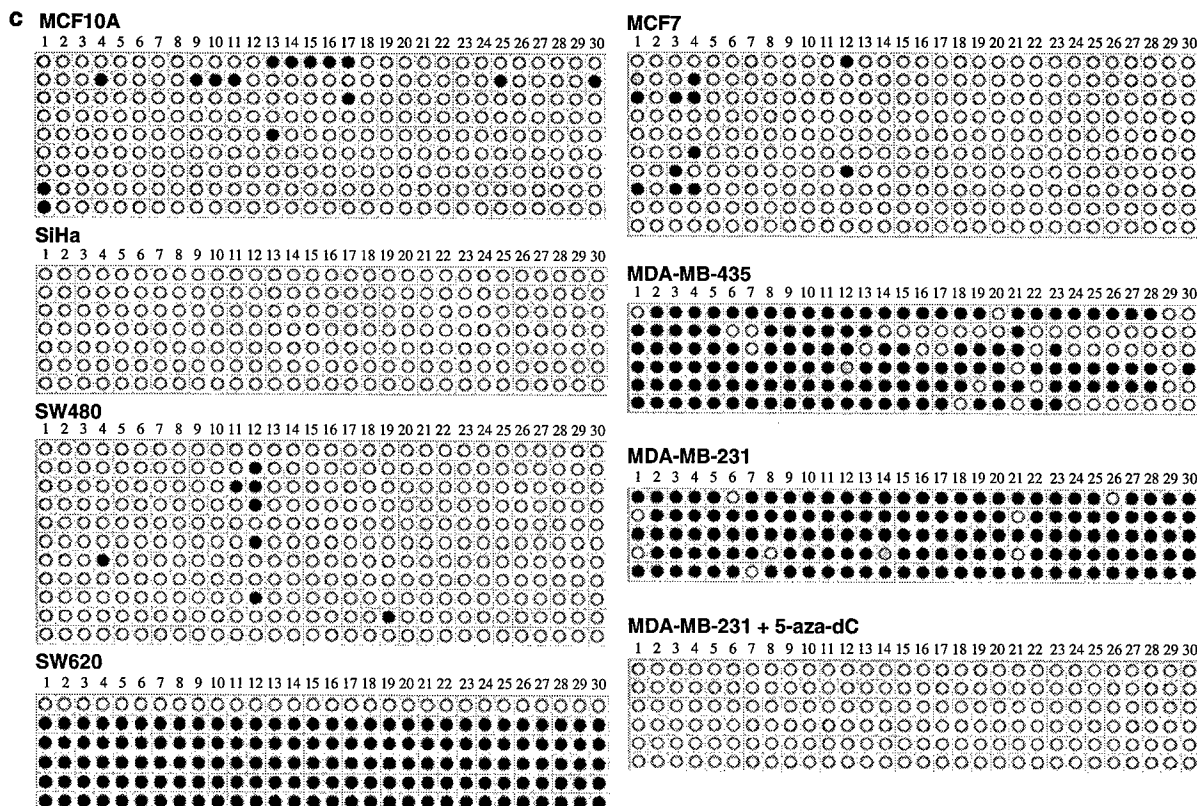
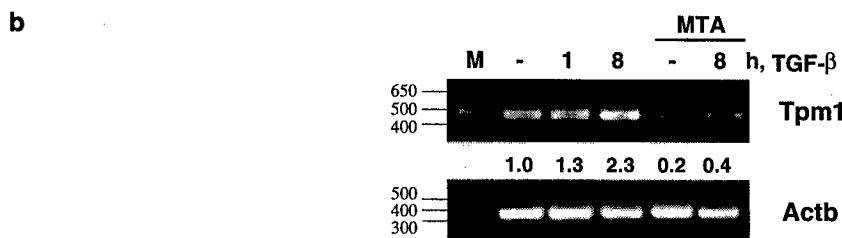
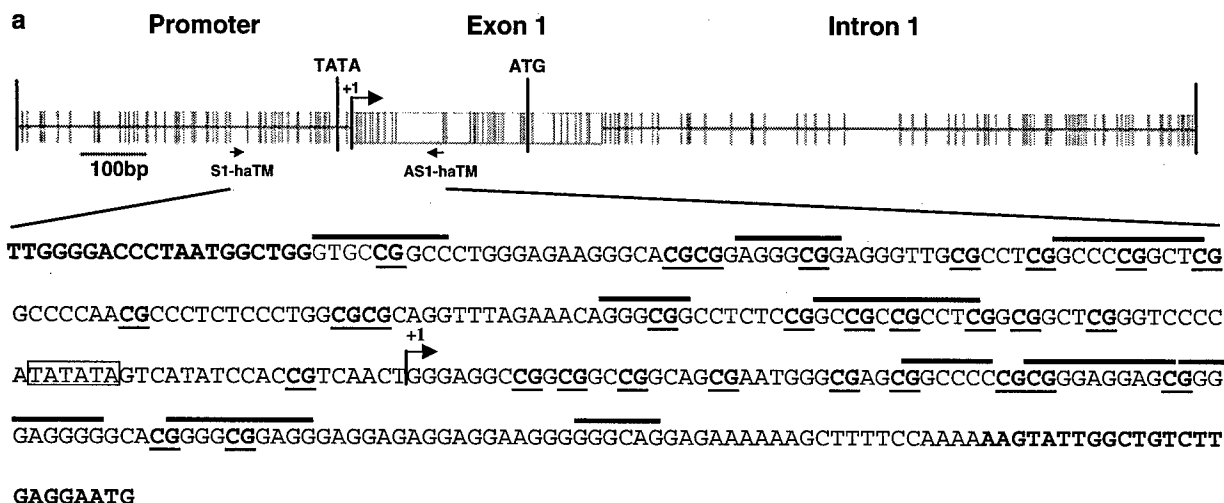


Figure 5 Expression of TM3 using Tet-Off system in MDA-MB-231 cells increases actin fibers and reduces cell migration. (a) Immunoblot of tropomyosin and α -catenin, a loading control, in MDA-MB-231-Tet-Off cell line incubated alone or with 1 μ g/ml doxycycline for 24 h. (b) Removal of doxycycline increases actin microfilament fibers in MDA-MB-231-Tet-Off cells. Scale bar, 20 μ m. (c) Transwell migration assay of the Tet-Off cells incubated alone or with 2 μ g/ml doxycycline for 16 h. The cells that had migrated through pores to the lower surface were stained with Diff-quick stain. (d) Average amount of cells per field. Cells were counted from five random fields at $\times 200$ magnification. Assays were carried out in duplicates

amplify a 328 bp fragment of TPM1 containing 30 CpG dinucleotides and several potential Sp1-binding sites (Figure 6a). gDNA samples from several cell lines were

extracted and treated with bisulfite. DNA fragments were amplified using bisulfite-treated DNA samples, subcloned, and both DNA strands were sequenced. The



bisulfite modification was complete in all reactions, as all cytosine residues not associated with CpG dinucleotides were converted to thymine residues (data not shown). The methylation status of 30 CpG dinucleotide sites in cell lines is shown in Figure 6c. DNA-bisulfite-sequencing showed a nearly complete methylation of CpG sites in MDA-MB-231 cells and a high degree of methylation in MDA-MB-435 cells (Figure 6c). The level of DNA methylation inversely correlated with expression level of TPM1 mRNA in these cells (Figure 6c and 2a). 5-aza-dC-treatment of MDA-MB-231 resulted in demethylation of cytosine residues (Figure 6c) and increased TPM1 expression (Figure 2c, 3b). Colon cancer SW480 cell line showed a low level of DNA methylation and also expressed TPM1 mRNA (compare Figure 6c and 2d). Metastatic SW620 cell line showed a high level of DNA methylation (Figure 6c) and very low levels of TPM1, which was upregulated by 5-aza-dC treatment (Figure 2d).

Thus, high level of methylation of TPM1 promoter region correlates with low TPM1 expression and demethylation of DNA upregulates TPM1 expression in metastatic cell lines.

Discussion

The molecular mechanism(s) underlying the prometastatic conversion of the TGF- β function at the late stages of tumorigenesis is not well understood (Derynck *et al.*, 2001; Roberts and Wakefield, 2003). TGF- β function in development of highly invasive and metastatic tumors has been associated with induction of EMT, cell migration and activity of matrix proteinases (Derynck *et al.*, 2001; Wakefield and Roberts, 2002). Recent studies have shown that stimulation of cell migration by TGF- β inversely correlates with expression of HMW tropomyosins encoded by TPM1 and TPM2 genes involved in formation of stable actin filaments (stress fibers) (Bakin *et al.*, 2004). Here, we report that TPM1 promoter hypermethylation correlates with a reduced expression of tropomyosins and inability of TGF- β to induce stress fibers in metastatic carcinoma cells.

Tropomyosins are actin-binding proteins involved in stabilization of actin filaments and control of cell motility (Pawlak and Helfman, 2001). Although the role of tropomyosins in muscle contraction is well

established, their role in non-muscle cells is less clear. Several lines of evidence suggest that HMW tropomyosins encoded by TPM1 (α -TM) and TPM2 (β -TM) genes may contribute in tumor suppressor activity of TGF- β : (i) HMW tropomyosins are required for TGF- β -mediated formation of stress fibers and inhibition of cell migration (Bakin *et al.*, 2004); (ii) oncogenic Ras and Src downregulate HMW tropomyosins leading to disruption of actin stress fiber filaments (Hendricks and Weintraub, 1981; Leonardi *et al.*, 1982) and ectopic expression of HMW tropomyosins in Ras- or Src-transformed cells restores stress fibers and reduces cell motility and cell growth (Braverman *et al.*, 1996; Gimona *et al.*, 1996; Janssen and Mier, 1997); and (iii) high-grade tumors of breast, prostate, bladder, and brain express significantly lower levels of HMW tropomyosins compared to normal tissues (Franzen *et al.*, 1996; Wang *et al.*, 1996; Hughes *et al.*, 2003; Raval *et al.*, 2003; Pawlak *et al.*, 2004).

In this study, a marked reduction in HMW tropomyosin was found in metastatic breast and colon cancer cell lines (Figure 2). Low tropomyosin levels in metastatic breast cancer MDA-MB-231 cells correlated with a reduced stress fiber formation in response to TGF- β (Figure 1). Recently, we have reported that Ras-MEK signaling decreases TPM2 expression and stress fiber formation in MDA-MB-231 cells (Bakin *et al.*, 2004). The current study showed the absence of basal and TGF- β 1-regulated expression of the TPM1 gene in MDA-MB-231 cells, although these cells express TGF- β receptors and Smad signaling components (Dumont *et al.*, 2003). TPM1 mRNA expression is also reduced in metastatic breast cancer MDA-MB-435 cell line and in metastatic colon cancer SW620 cell line (Figure 2). Interestingly, colon cancer SW480 cell line established from the primary lesion of the same patient expressed higher level of TPM1 compared to metastatic SW620. These findings show that low level of TPM1 expression correlates with metastatic phenotype of tumor cells.

Treatment of metastatic breast and colon cancer cell lines with demethylating agent 5-aza-dC increased TPM1 expression (Figure 2). Previously, one study has reported that 5-aza-dC treatment upregulates expression of TPM2, but not TPM1, in MCF7 and MDA-MB-231 cell lines (Bharadwaj and Prasad, 2002), while another group showed that both TPM1 and TPM2 are upregulated in fibrosarcoma HT1080 cells (Shields *et al.*, 2002). Both of the studies used Northern blot probes that do not distinguish TPM1 and TPM2 genes. In the

Figure 6 The human TPM1 promoter and methylation of a CpG island. (a) Depiction of the 1829 bp CpG island within the human TPM1 promoter. The transcription initiation site is shown as +1. The TATA-like sequence is boxed. Sp1-like sites are depicted above the sequence. The CpG dinucleotides are in bold font and underlined. The positions of the bisulfite-specific primers relative to the promoter and exon 1 are shown. (b) Effect of mithramycin A on Tpm1 expression in response to TGF- β 1 in NMuMG cells examined by RT-PCR. β -actin (Actb) is a control. (c) Methylation analysis of 30 CpG pairs from a 328 bp region of the human TPM1 CpG island in human cell lines and tissue samples. The presence of methylated cytosine at CpG sites in DNA from cancer-derived cell lines and 5-aza-dC-treated MDA-MB-231. PCR fragments of bisulfite treated DNA were cloned into pGEM-Teasy and sequenced in both directions. The sequencing results for the individual CpG sites are depicted. Each horizontal line depicts a single clone and black circles represent methylated cytosines; open circles: unmethylated cytosines; and gray circles: equivocal data. CpG sites are positioned with respect to the +1 (Figure 4): -162, -143, -141, -134, -124, -119, -113, -108, -99, -85, -83, -64, -55, -51, -48, -43, -40, -35, -8, +8, +11, +15, +21, +29, +33, +40, +42, +51, +64, +69

current study, RT-PCR with TPM1 and TPM2 gene-specific primers was used to show that TPM2 transcripts are expressed at comparable levels in all tested breast cancer and normal cell lines, whereas TPM1 is down-regulated in metastatic MDA-MB-231, MDA-MB-435 and SW620 cell lines (Figure 2). 5-aza-dC treatment significantly enhanced TPM1 mRNA levels but not TPM2 in all three metastatic cell lines (Figure 2). These data suggest that methylation may not regulate TPM2 expression directly. Demethylation of MDA-MB-231 cells restored the ability of TGF- β to induce TPM1 expression, stress fibers and reduced cell migration (Figures 3 and 4), suggesting that silencing of TPM1 by DNA methylation reduces stress fibers and enhances cell motility.

Importantly, expression of TPM1 gene product in MDA-MB-231 cells using Tet-Off system increased stress fibers and reduced cell migration (Figure 5). However, TGF- β can induce stress fibers even in the absence of TPM1 expression (Bakin *et al.*, 2004). A forced expression of either TPM1 or TPM2 can reduce anchorage-independent cell growth (Boyd *et al.*, 1995; Braverman *et al.*, 1996; Masuda *et al.*, 1996) and TPM2 can induce anoikis (Raval *et al.*, 2003). These facts suggest that a critical threshold level of HMW tropomyosins encoded by TPM1 and TPM2 genes is required for stress fiber formation in response to TGF- β in epithelial cells.

Database-mining revealed in both human and mouse TPM1 genes a CpG island within a proximal promoter region, which includes the TATA box, a putative transcription start site, and several Sp1-like sites (Figure 5). Bisulfite sequencing showed a high degree of cytosine methylation within the -186 + 142 region of the TPM1 promoter in metastatic cell lines, but not in cell lines established from non-neoplastic (MCF10A) or primary tumors (MCF7, SiHa and SW480) (Figure 5c). Treatment with demethylating agent of metastatic breast cancer MDA-MB-231 cells removed methylation and upregulated TPM1 expression, suggesting an inverse correlation between TPM1 expression and hypermethylation of the TPM1 promoter.

Basal expression of TPM1 appears to not require Smad4 since TPM1 is expressed in Smad4-null MDA-MB-468 cells (Figure 2). However, Smad4 is required for TGF- β induction of TPM1 (Bakin *et al.*, 2004). Experiments with MTA suggest that Sp1 or Sp1-like factors may contribute to basal and TGF- β -mediated expression of TPM1 (Figure 5b). Thus, TPM1 may represent a group of TGF- β target genes that are regulated by both Sp1 and Smads including p21Cip1 (Moustakas and Kardassis, 1998), α 2 collagen (Chung *et al.*, 1996; Greenwel *et al.*, 1997) and p15Ink4B (Feng *et al.*, 2000). Interestingly, Sp1-mediated expression of p21Cip1 is sensitive to DNA methylation (Zhu *et al.*, 2003). Thus, cytosine methylation in Sp1-like sites of the TPM1 promoter may contribute in suppression of basal and TGF- β -induced expression of TPM1.

In summary, our studies revealed that hypermethylation of the TPM1 promoter associates with a reduced TPM1 expression in metastatic cell lines. Suppression of

TPM1 may underlie failure of TGF- β to induce stress fibers and to inhibit migration in metastatic cells. Thus, epigenetic suppression of tropomyosin-mediated stress fibers may represent an essential characteristic of prometastatic changes in TGF- β function and restoration of the stress fiber response is a potential strategy for antimetastatic therapy.

Materials and methods

Cell lines

Human cell lines of breast carcinoma MCF7, MDA-MB-231, MDA-MB-435, MDA-MB-453, MDA-MB-468, BT549, SKBr3 and T-47D, normal breast MCF10A, cervical carcinoma SiHa, colon carcinoma SW480 and SW620, lung carcinoma A549 and mouse mammary epithelial cell line NMuMG were purchased from American Tissue Culture Collection (ATCC). Cell lines were cultured as recommended by ATCC.

Generation of Tet-Off MDA-MB-231 cells

Human breast carcinoma MDA-MB-231 cells (ATCC) were cotransfected with pBabe-Puro and pTA-IRES-Neo plasmid (tTA, tet activator, IRES, internal ribosome entry site) (Yu *et al.*, 1999). Puromycin-resistant clones exhibiting TGF- β responses equal to parental cells were selected in the presence of 1 μ g/ml of puromycin. Two clones with a tight regulation of the tet-responsive reporter plasmid pBI-MCS-EGFP (Yu *et al.*, 1999) were chosen to generate inducible cell lines. cDNA for rat tropomyosin 3 isoform (Gimona *et al.*, 1996) was subcloned in pBluescript II KS(+) (Stratagene) at *Bam*HI/*Xba*I sites and then shuttled into pTRE2hyg (BD Biosciences Clontech, Palo Alto, CA, USA) at *Not*I/*Sa*I sites to generate pTRE2hygTM3. The MDA-MB-231 Tet-Off cell lines were transfected with pTRE2hyg-TM3 encoding untagged rat TM3 and cells retaining TGF- β responses were selected in the presence of 200 μ g/ml hygromycin and 2 μ g/ml doxycycline. Induction of TM3 expression by removal of doxycycline was confirmed by immunoblotting with TM311 antibody.

Antibodies, plasmids and other reagents

TGF- β 1 was obtained from R&D Systems. The following antibodies were used: mouse monoclonal to Smad2/3 (BD Transduction Laboratories, BD Biosciences); rabbit polyclonal to hemagglutinin (HA) epitope (Santa Cruz Biotechnology, Inc.); mouse monoclonal TM311 to tropomyosin (Sigma); to phospho-Smad2/3, phospho-ERK1/2 and total ERK1/2 (Cell Signaling). Phalloidin-Alexa Green and phalloidin-Texas Red were from Molecular Probes.

Isolation of DNA and RNA

DNA was isolated using the high salt extraction method (Miller *et al.*, 1988). RNA was isolated as described in Bakin and Curran (1999).

5-aza-2'-deoxycytidine and TGF- β 1 treatment of cell lines

MDA-MB-231 cells (1×10^6) were seeded into 25 cm² flasks and were treated with 2 μ M 5-aza-2'-deoxycytidine (5-aza-dC) for 48 h. Cells were washed with PBS, and were incubated in fresh media for a further 48 h. Cells were seeded into 25 cm² flasks and 2 μ M 5-aza-dC treatment was repeated, followed by

the addition of media containing 5% serum. The next day, to cells requiring TGF- β 1 treatment, 2 ng/ml TGF- β 1 was added for 24 h. DNA, RNA and protein were extracted from samples. MCF10A, MCF7 and MDA-MB-435 were treated with 5-aza-dC as previously described (Li *et al.*, 2004).

Mithramycin A (MTA) treatment of cells

NMuMG cells (6×10^5) were seeded in media containing 5% serum. Cells were subsequently left untreated or treated with both 200 μ M MTA and 2 ng/ml TGF- β 1 for 1 or 8 h. Total RNA was analysed by RT-PCR for expression of TPM1 and β -actin.

RT-PCR

Amplification of transcripts was performed using 50 ng of total RNA and the one-step RT-PCR system from Invitrogen according to the manufacturer's protocol, using primers designed with the assistance of Primer3 (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi). PCR in the one-step system were performed at an annealing temperature of 55°C within a linear range of amplification. Primer sequences: human TPM1, GenBank Acc# NM_000366, forward: 5'-GCTGGTGTCACTGCAAAAGA-3', reverse: 5'-CTGCAGCCATTAATGCTTTC-3'; human TPM2, GenBank Acc# NM_003289, forward: 5'-AAGGAGGCCCCAGGAGAAACT-3', reverse: 5'-CTTCCTTCAGCTGCATCTCC-3'; human β -actin, GenBank Acc# NM_001101, forward: 5'-GCTCGTCGTCGACAACGGCTC-3', reverse: 5'-CAAACATGATCTGGGTCATCTTCTC-3'; mouse Tpm1, GenBank Acc# NM_024427, forward: 5'-GCTGGTGTCACTGCAAAAGA-3', reverse: 5'-CCTGAGCCTCCAGTGACTTC-3'; and mouse β -actin, GenBank Acc# NM_007393, forward: 5'-GCTGGTGTGCGACAACGGCTC-3', reverse: 5'-CAAACATGATCTGGTTCATCTTTC-3'. Forward and reverse primers for human TPM1 and TPM2 genes cover amino acids 43–48 and 205–209 of TPM1, and 65–71 and 145–150 amino acids of TPM2, respectively. The associated product sizes for human TPM1, TPM2, β -actin and mouse Tpm1 and β -actin were 506, 258, 353, 506 and 352 bp respectively.

Transient transfection

Plasmids encoding rat HA-tagged TM2 and TM3 isoforms (Gimona *et al.*, 1995) were obtained from David Helfman (CSHL, Cold Spring Harbor, NY, USA). In total, 10 μ g plasmid was transfected with FuGENE 6 (Roche) into MDA-MB-231 cells, according to the manufacturer's instructions.

Identification of CpG island within the TPM1 promoter and bisulfite sequencing

The promoter region of human and mouse TPM1 genes were initially predicted using BLAST against rat exonic sequences, and then analysed in BLAT at <http://genome.ucsc.edu/cgi-bin/hgBlat> (Genome Bioinformatics Group, University of California, USA). Both human and mouse CpG islands were identified in BLAT. Primers S1-h α TM (5'-TTGGGGATTTAATGGTTGG-3') and AS1-h α TM (5'-CATTCCTCAAAA-CAACCAATACTT-3') amplifying bisulfite-treated DNA were designed with Primer3. Bisulfite treatment was performed as previously described (Varga *et al.*, 2004). PCR reactions were carried out under the following conditions: 3 min at 94°C, followed by 40 cycles of 30 s at 94°C, 30 s at 50°C, and 55 s at 72°C, followed by a final elongation for 7 min at 72°C before holding at 4°C. PCR products were resolved on a 1% agarose gel, purified with a PCR purification kit (Marligen Biosciences,

USA) and cloned into pGEM-Teasy (Promega, USA). Clones were isolated and sequenced in both directions and CpG sites were analysed for methylation.

Immunoblot analysis

Cells were incubated in medium containing 5% serum for 24 h prior to treatment with 2 ng/ml TGF- β 1. Cells were lysed in buffer containing 20 mM Tris, pH 7.4, 137 mM NaCl, 1% NP-40, 10% glycerol, 20 mM NaF, 1 mM Na orthovanadate, 1 mM PMSF, 2 μ g/ml aprotinin and 2 μ g/ml leupeptin. Immunoblot analyses of whole-cell extracts were performed as described (Bakin *et al.*, 2002).

Immunofluorescence microscopy

Cells (10^5 cells/well) were grown in DMEM containing 5% FBS on glass coverslips (22 \times 22 mm) for 24 h before treatment with 2 ng/ml TGF- β 1. Cells were fixed with 4% paraformaldehyde and stained as described (Bakin *et al.*, 2002). Actin filaments were stained with phalloidin-Alexa Green or phalloidin-Texas Red and tropomyosins were visualized using TM311 antibody. Fluorescent images were captured using a Nikon TE2000-E inverted microscope.

Transwell migration

The MDA-MB-231-Tet-Off-TM3 cells were incubated in the presence or absence of 2 μ g/ml doxycycline for 24 h prior to the migration assay. Cells (1×10^5 cells/well) were placed in DMEM/1% FBS in the upper chamber of 5- μ m pore (24-well) transwells (Costar, High Wycombe Bucks, UK) and incubated alone or with 2 μ g/ml doxycycline for 16 h. The cells were fixed in 100% methanol and cells remaining at the top of the polycarbonate membrane were removed with cotton swabs. The cells that had migrated through pores to the lower surface were stained with Diff-quick stain (VWR Scientific). Membranes were mounted on 25 \times 75-mm² microslides. Cells were counted from five random fields at \times 200 magnification. Experiments were performed in duplicates.

Wound closure assay

MDA-MB-231 cells ($1-2 \times 10^5$ cells/well) were seeded in 12-well plates. Cells were incubated in serum-free medium for 24 h prior to wounding. The wounds were made by scraping with plastic tip across the cell monolayer. The wounded cells were treated or untreated with 2 ng/ml TGF- β 1. Phase contrast images were recorded at the time of wounding (0 h) and 20 h thereafter. Experiments were repeated with two independent 5-aza-dC treatments.

Abbreviations

TGF- β , transforming growth factor beta; MAPK, mitogen-activated protein kinase; TPM, tropomyosin; 5-aza-dC, 5-aza-2'-deoxycytidine.

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